

EXH 2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Kaare M. GAUTVIK et al.
Title: *Production of Human Parathyroid
Hormone From Microorganisms*
Appl. No.: 08/340,664
Filing Date: November 16, 1994
Examiner: L. Spector
Art Unit: 1647

DECLARATION OF KAARE M. GAUTVIK, M.D.

PURSUANT TO 37 C.F.R. § 1.132

Mail Stop NON-FEE AMENDMENT
Commissioner for Patents
PO Box 1450
Alexandria, Virginia 22313-1450

Sir:

I, KAARE M. GAUTVIK, declare as follows:

1. I am a co-inventor of the above-captioned application.
2. I am a citizen of Norway residing at Bregnevn 3, 0875 Oslo, Norway. I am fluent in English. My curriculum vitae is attached hereto as Exhibit A.
3. The isolated hPTH described by Brewer (U.S. Patent No. 3,886,132) in column 2, lines 3-11 is less than 90% pure.
4. Brewer's ability to sequence the first 34 amino acid residues of his final hPTH preparation by Edmond degradation does not mean that Brewer's final hPTH preparation was

more than 90% pure. To the contrary, Brewer's sequence contained 3 serious amino acid errors indicating that Brewer's final hPTH preparation is less than 90% pure.

a. Brewer's Purification and Edmond Degradation Method

5. Brewer isolated hPTH from dried, defatted parathyroid tissue as described in column 2, lines 3-13. Brewer indicates that the final hPTH preparation was analyzed by disc gel electrophoresis and monitored by radioimmunoassay. However, Brewer fails to provide a picture of the gel or any other documentation regarding the purity of the final hPTH preparation. As a result, a determination of the purity of the final hPTH preparation can be based only on an analysis of the results of Brewer's N-terminal sequencing of the first 1-34 amino acid residues from the intact hPTH of 1-84 amino acid residue protein/peptide using Edman degradations employing the Beckman Sequencer, Model 890B. The year that the 890B model was introduced is believed to be 1973.

6. Brewer's Edman degradation involved a Beckman sequencer in which phenylthiohydantoin amino acids were identified by regeneration to the constituent amino acid by hydrolysis with hydroiodic acid. Identification of the amino acid derivatives was carried out by gas liquid chromatography and mass spectrometry. See column 2, lines 23-39, of Brewer. Brewer degraded 350 nanomoles of the final hPTH preparation on the Beckman Sequencer using a single cleavage of heptofluorobutyric acid at each degradation. The results of the degradation of the first 34 residues is shown in Figure 1. See column 2, lines 55-59.

b. The amino acid sequence reported for the first 34 amino acids of Brewer's final hPTH preparation contains 3 mistakes

7. The sequence results of Brewer's Edman degradation contained 3 mistakes, namely at position 22 (gln instead of glu), in position 28 (lys instead of leu) and in position 30 (leu instead of asp). The probable conclusion as to why Brewer failed to obtain a correct sequence of the first 34 amino acids of hPTH, is that Brewer's final hPTH preparation was impure - containing a significant amount of other peptides/proteins which seriously disturbed and blurred the results of the Edman degradation analysis. This is because the presence of contaminating proteins in Brewer's final hPTH preparation, dramatically increased the chances to obtain a faulty sequence for hPTH.

- c. **The presence of more than one peptide/protein in the original mixture leads to greatly increased probability for incorrect identification of amino acid residues**

8. A major problem relating to correct identification of each amino acid residue via the Edman degradation sequence method is the presence of more than one peptide/protein in the original mixture. A feature of the Edman degradation sequencing method is that the material that is being analyzed is consumed during the process, rendering stepwise identification of each amino acid more uncertain as sequencing moves away from the start N-terminal residue. Another feature of the method is the accumulation of blocked peptides during the process which obscure the obtained results. These two features, along with impurities in the starting material, cause the absolute and relative amounts of non-natural hPTH molecules to increase substantially as a function of degradation steps (cycles), thus reducing the accuracy and fidelity of the amino acid derivative chromatogram readings. See the graph on the left side of the of the Beckman User Manual, "Determining Sequencer Sensitivity" (1983) (Exhibit B), which shows reduction in yield of each amino acid (reflecting the true loss of substance) as a function of the number of cycles.

9. The graph on the left side of the Beckman User Manual shows sequencing of apomyoglobin on a more modern Beckman instrument (developed about 10 years later than the Model 890B utilized by Brewer). It is noted that "PTH-amino acid" as used in the Beckman reference, is shorthand nomenclature used by those of skill in the art to designate an amino acid of the method employed, and does not refer to parathyroid hormone. The graph depicts the quantitative recovery (yield) of different amino acid residues (shown in capital letters) as a function of degradation steps (cycles). As the amount of available material that is being analyzed is reduced, the yield of a given amino acid is correspondingly reduced, rendering contaminating proteins and other impurities to exert an increasingly negative effect on the accuracy of the readings of the chromatograms. For example, after cycle 20 in the apomyoglobin case, the yield of recovery of the next amino acid residue is less than 50% of the initial yield of material (the initial yield of apomyoglobin was 63.8%). This technology represents at least a 10 year advancement in technology from the filing date of Brewer et al., suggesting a poorer yield for Brewer's material by cycle 20.

10. Typically, sequencing mistakes start to occur after the first 15-20 amino acids in the peptide are analyzed by Edmond degradations. In the initial cycles of Edmond degradations, when the amount of material that is being analyzed is large, the peaks in the chromatogram representing the major component of the material (hPTH) are larger than the peaks in the chromatogram representing the other components in the material (contaminants). (See the chromatograms on the right side of the page of the Beckman User Manual, "Determining Sequencer Sensitivity" (1983)) (Exhibit B). See especially the reduction in leu peak height given as recovered picomoles (yield), which falls dramatically after cycle 10. Note also that at line 2, page 2 of the Beckman User Manual, it strongly recommends that "[b]ackground subtraction or 'data enhancement' (if any) should be indicated" (line 2 page 2). "Background" refers to chromatographic noise due to impurities of all kind (see above). It was not an acceptable standard in 1975, nor is it acceptable today, to fail to include copies of genuine chromatograms of amino acid derivative separation for sequences determined by Edmond degradation. However, Brewer did not include copies of his chromatograms in the cited reference.

11. In the later cycles of Edmond degradation, when the amount of correct available/remaining material that is being analyzed is much smaller, the peaks in the chromatogram representing the major component (hPTH) are comparable in size to the peaks in the chromatogram representing the other components in the material (contaminants). Therefore, the scientist is forced to "guess" which peak represents the major amino acid derivative in the hPTH sequence and which peak represents the other components (contaminants). The fact that the hPTH sequence disclosed by Brewer in Figure 1 contains 3 mistakes (at residues 22, 28 and 30) suggests that Brewer's final hPTH preparation contained contaminants and is clearly less than 90% pure.

d. Gas liquid chromatography causes separation difficulties with asparaginy, glutaminy, and lysyl derivatives

12. As discussed above, after performing Edmond degradation, Brewer identified amino acid derivatives by gas liquid chromatography and mass spectrometry. However, as described on page 281 of *Protein Sequence Determination* (2nd revised and enlarged edition)

(1975), Edited by Saul B. Needleman (Springer-Verlag, Berlin-Heidelberg-New York) (Exhibit C), the use of gas liquid chromatography is known to cause separation difficulties with several amino acid residues including asparaginy, glutaminy, lysyl and other amino acid derivatives.

13. The mistakes in Brewer's sequence involve asparaginy, glutaminy, and lysyl derivatives. To at least partly overcome these separation difficulties, Brewer could have employed other liquid phases and modifying reagents. However, Brewer does not indicate that such agents were used.

e. Brewer started with a much less than recommended amount of hPTH for sequencing.

14. Brewer assumes that 350 nanomoles of his final hPTH preparation were degraded on the Beckman Sequencer. As noted on page 34, line 14, of the specification, the molecular weight of hPTH is 9000 Daltons. Therefore, if Brewer's final hPTH preparation was truly "pure" hPTH, then this would mean that Brewer isolated at least 3.15 mg of hPTH (as described below, it is highly likely that Brewer's final hPTH preparation contained much less than 3.15 mg of intact, pure hPTH). 3.15 mg of protein, however, is insufficient for obtaining accurate sequence results, as clearly stated in Hermodson et al, *Biochemistry*, 11:4493-4501 (1972) (see page 4497, second column, next last paragraph) (Exhibit D), which suggests a recommended sample size for the initial sequencing cycle of 7-10 mg.

15. An insufficient amount of starting material makes sequencing accuracy and fidelity in interpreting chromatograms much more susceptible to serious mistakes. Brewer's amount of starting material was insufficient, and as a result, the amino acid sequence Brewer reported for the first 34 amino acids of Brewer's final hPTH preparation contained 3 mistakes.

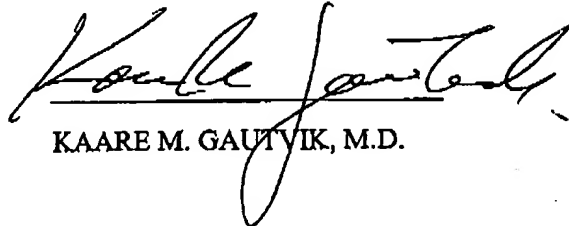
f. It is unlikely that Brewer could have isolated 3.15 mg of hPTH from parathyroid gland adenomas that was more than 90% pure

16. As noted above, 350 nanomoles of hPTH would be about 3.15 mg of hPTH. Brewer fails to specify the initial weight of the parathyroid tissue from which Brewer isolated

the final hPTH preparation. However, if 1% of the total weight of the parathyroid tissue comprised hPTH (this is most likely an over estimation), then in order for Brewer to have isolated 3.15 mg of hPTH, he would have had to start out with 315 mg of parathyroid tissue. Since adenomateous hPTH gland weighs approximately 10-20 mg, this would mean that Brewer isolated hPTH from probably more than 16 glands. It would be impossible for a skilled artisan to purify 3.15 mg of hPTH from 16 glands. Therefore, a more likely explanation is that Brewer's final hPTH preparation contained a significant amount of contaminants and was less than 90% pure.

17. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 07.27.04


KAARE M. GAUTVIK, M.D.

**CURRICULUM VITAE
RELEVANT PUBLICATIONS
KAARE M. GAUTVIK, M.D., PH.D, CHIEF CONSULTANT**

exh A

Personal and marital status:

Name: Kaare M. Gautvik
 Home address: Bregnevn. 3, 0875 Oslo, Norway
 Business address: Institute of Medical Biochemistry, University of Oslo,
 P.O.Box 1112 Blindern, 0317 Oslo, Norway
 Telephones: 47-22851055 (work); 47-22235137 (home)
 Date and place of birth: 11th of December 1939 in Oslo.
 Social Security: No.: 111239.39311
 Married to: Vigdis Teig Gautvik, date of birth: 24th of March 1947
 Children: Lars Erlend Sakrisvold Gautvik, date of birth: 9th of January 1964
 Silja Marie Sakrisvold Gautvik, date of birth: 31th of March 1973, Ole Martin Teig Gautvik, date of birth: 21th of January 1982

Education and Clinical Specialities:

1. August 1958-June 64, Medical School at the University of Oslo.
2. 1967-69, Courses in mathematics involving geometry, statistics and mathematical analysis.
3. May 1970, Disputation for the medical doctor degree at the University of Oslo.
4. 1985, Specialist in clinical chemistry, and physiology and nuclear medicine.
5. 1986, Specialist in occupational health medicine.

Employment:

1. June 1964-June 1965, working at Tromsø University Hospital at medical and surgical departments.
2. July 1965 until December 1965, working as a general practioner in Sjøvegan, Troms.
3. One year military service as a major in The Norwegian Air Force, working mainly at the Norwegian Institute for Aviation and Space Medicine.
4. From 1967, position as post-doctoral researcher at The Institute of Physiology, University of Oslo.
5. From September 1969, promoted to Assistant Professor at the University of Oslo, Institute of Physiology.
6. Leader and responsible for clinical and experimental endocrinological laboratory of Institute for Surgical Res., The National Hospital, Oslo, from 1973-89.
7. From 1976-1978, training as a specialist in clinical chemistry at the Norwegian Radium Hospital, Oslo.
8. From August 1983 appointed to full professor at the Institute of Medical Biochemistry, Medical Faculty, University of Oslo.
 (At the same time receiving offers of professor chairs at the Institute of Physiology, Medical Faculty and at the Institute of Physiology and Biochemistry, Faculty of Odontology).
9. From Jnuary 2002 employment as senior consultant at Department of Clinical Chemistry, Labaratory Division, Ulleval University Hospital and professor II at the University of Oslo.

Post-doctoral training abroad:

1. For three months in 1967, I worked as a lecturer at the Department of Physiology, Medical School, Birmingham University, England.
2. From August 1971 to July 1973 in receipt of Fogarty international post-doctoral fellowship at the Department of Pharmacology, Harvard School of Dental Medicine and Harvard Medical School.
3. 1980, 4 months Visiting Professor at Institute of Genetics, BMC, Uppsala University, Sweden.
4. 1995/96, 12 months Visiting Research Professor, The Scripps Research Institute, Dept. Mol. Biology, La Jolla, San Diego, USA.
5. 1997, 3 months Visiting Research Professor, The Scripps Research Institute, Dept. Mol. Biology, La Jolla, San Diego, USA.

Teaching responsibility:

1. One year teaching in aviation medicine for medical personnel and pilots.
2. I have given lectures and courses for medical students in following subjects:
 Haematology, kidney physiology, endocrinology, circulation, respiration and gastrointestinal physiology.
 From 1983 organized and given lectures and courses in molecular genetics at undergraduate and postgraduate level for students in medicine and sciences.
3. Organized interfaculty advanced courses within molecular endocrinology.
4. Lectures have been given in the following subjects at post-doctoral courses:
 Diseases of the thyroid gland (1973); Regulation of circulation in the gastrointestinal system (1973); Local hormones (1975); Endocrinology (annually from 1978); Tumour markers (1979); Calcium metabolism (annually from 1980); Ligands for peptide hormone-receptors, and Nuclein acid biochemistry (1984); TRH-receptors in prolactin-producing cells (1985). Molecular biology in medical research (yearly from 1983). Biochemical analysis on bone material (1991).
5. Invited lectures: Several places in the U.S., in Sweden, in Finland, and in England, as well as different places in Norway, a total of 37 as of 1995.

6. Chief organizer of post graduate scientific courses for the Medical Faculty at University of Oslo, 1986-1991.
7. Organizer of international scientific meetings within the frame of the following societies: Acta Endocrinologica (European International Endocrine Society), The Scandinavian Physiology and Pharmacology Meetings, and the Norwegian Biochemical Society.
8. Introduced teaching in Molecular Biology for students at the Medical Faculty, Oslo.
9. Invited as Symposium Lecturer at international meetings in physiology and endocrinology and molecular biology as exemplified below.

Examples of specially invited symposium lectures:

1. February, 1990: "Production of recombinant human parathyroid hormone in E.coli and Saccharomyces cerevisiae and its potential use as drug in osteoporosis" by Kaare M. Gautvik, Eli Lilly Co., Indianapolis, USA, in a Biotechnology meeting.
2. June, 1990: Symposium lecturer and organizer: "Hormone receptors and cellular signal transduction. The XXII Nordic Congress in Clinical Chemistry, Trondheim, Norway.
3. July, 1990: Symposium lecturer: "Transmembrane signal systems involved in the regulation of prolactin secretion by hypothalamic peptide hormones in cultured pituitary cells. 2nd European Congress of Endocrinology, Ljubljana, Yugoslavia.
4. July, 1990: Symposium lecture: "Successful cloning and production of human parathyroid hormone (hPTH) in yeast as a secretory product". 5th European Congress on Biotechnology, Copenhagen, Denmark. (Unable to attend, and the lecture was held by cand.scient. Sjur Reppe).
5. August, 1990: Symposium lecture: "Processing and stability of human parathyroid hormone produced in E.coli and S.cerevisiae studied by *in vitro* mutagenesis". Workshop/Symposium on site-directed mutagenesis and protein engineering, Tromsø, Norway.
6. December, 1990: Invited by Professor Guo Hui-Yu, Guangzhou, China and Professor G.L. French, Hong Kong. Lecture entitled: "Expression of human parathyroid hormone as a secretory protein in prokaryotic and eukaryotic microorganisms". The Second International Conference on Medical Microbiology and Biotechnology Towards 2000, Guangzhou, China. (Did not attend as a protest against the punishment of the students rebellion in Peking).
7. January 1991: Invited to a Workshop by Dr. Stephen Green, Central Toxicology Laboratory, ICI, Alderly Park, Macclesfield SK10 4TJ, UK. Lecture entitled: "Synergistic effects of hormones and fatty acid on peroxisomal β -oxydation, enzyme activities and mRNA levels".
8. January 1991: Invited to a Protein Engineering Meeting by Professor Ian Campbell, Biochemistry Department, Oxford University, Oxford, UK. Lecture entitled: "Cloning and expression of human parathyroid hormone in microorganisms".
9. Invited by Professors T.T. Chen, D.A. Powers, B. Cavari, Maryland Biotechnology Institute, Baltimore, MD, to held a symposium lecture at the 2nd International Marine Biotechnology Conference, October 13-16, 1991, Baltimore, Maryland, USA. (Could not attend).
10. May 1991: Invited by Professor Jan Carlstedt-Duke, Karolinska Institutet, Huddinge, to held a lecture in the seminar series "Novum Lectures in Cellular and Molecular Biology".
11. January 1992: Invited by Professor Armen H. Tashjian, Department of Molecular and Cellular Toxicology, Harvard School of Public Health and Department of Biological Chemistry on Molecular Pharmacology, Harvard Medical School, Boston, USA. Lecture entitled: "Use of antisense RNA in delineation of the mechanism of action of G-coupled hormones".
12. August 1993: Invited by Norwegian Society of Chartered Engineers, The Blindern Conference. Lecture entitled: "Experience from industrializing basal research".
13. November 1993: Invited by Karolinska sjukhuset, Stockholm, to held a lecture at "Graduate course in molecular endocrinology - a problem oriented approach". The lecture is entitled: "Region specific actions of parathyroid hormone in target tissues".
14. February 1994: Invited by GBF, Gesellschaft für Biotechnologische Forschung mbH, Braunschweig. Lecture entitled: "Expression of human parathyroid hormone in microorganisms and animal cells with special reference to signal sequence efficacy and intracellular modifications".
15. September 1994: Invited by Professor K. Dharmalingam, Department of Biotechnology, Madurai Kamaraj University, India, to held a lecture in the symposium "Gene expression systems", XVth IUBMB, New Delhi. Lecture entitled: "Expression of human parathyroid hormone in microorganisms, insect cells, mammalian cells and as a milk protein in transgenic mice".
16. November 1994: Invited by Professor A. Taschjian Jr., Harvard School and Public Health, Boston, to held a lecture in a seminar. Lecture entitled: "Certain structural and functional characteristics of the human TRH receptor cDNA and mapping of the gene".
17. February 11-13, 1995: Cairns, Australia, Workshop on "Animal models in the prevention and treatment of osteopenia".
18. February 1995: Int. Meeting of Calcified tissue research, Melbourne, Australia.
19. May 1996: Dublin University Program. "How to identify patients at risk for development of osteoporosis".
20. September 1996: Lecture at Scripps Research Institute, San Diego. "Unique hypothalamic specific mRNAs expressed by molecular subtraction hybridization".
21. September 1996: Invited seminar at the Astra Research Center, Montreal. "Cloning and expression of human polypeptide hormones with biomedical potential".
22. November 1996: Invited lecturer, The Norwegian Rheumatological Society, Oslo, "PTH (parathyroid hormone) - The biochemical foundation for treatment of osteoporosis".
23. December 1996: Invited at Nordic Conference for Medical Treatment of Osteogenesis Imperfecta, Holmen Fjordhotel, Asker, Norway. "Characteristics of bone remodelling in patients with osteogenesis imperfecta".

24. January 1997: Invited lecturer at The Salgrenska Hospital in Sweden. "Characterization and functional analysis of novel hypothalamus genes as identified by directional tag subtraction".
25. 1998: Guest lecturer at Scripps Research Institute: "Hypothalamic calcium-calmodulin kinase-cloning and functional aspects".
26. February 1999: Only invited speaker from abroad at National Osteoporosis Congress in Rio de Janeiro, Brazil.
27. 2000: Lecture at NPS-Allelix company and Toronto University: "Parathyroid hormone regulated bone remodelling".
28. May 2000, Rio de Janeiro, Brazil. Member of the International Scientific Panel at the International Congress in Osteoporosis.

Honorary lectures and prizes:

1. In 1984 recipient of Professor Olav Torgersen's Prize and Memorial lecture. This prize and lecture was created by Professor Torgersen, the University of Oslo, who was one of the founders of the Society for Promotion of Cancer Research in Norway. Because he contributed with personal money, the prize and lecture had his name. The title of my lecture was: "The medullary thyroid carcinoma: a special type of familial and hormone producing cancer".
2. In 1984 I was given the international science prize called The Nordic Insulin Prize instituted by Professor Jacob E. Poulsen, who worked at the University of Copenhagen. This prize is given within endocrinology and the candidate is chosen from all the countries in Northern Europe. The money was donated by the Insulin Laboratory now the company Novo-Nordisk. At that time, only one Norwegian had previously received this prize. The prize was given for my studies regarding how hormones exerted their biological actions in target cells.
3. The Gunnerus Prize was given in 1986 by the Royal Society of Norwegian Scientists. This is a prize which is given to a scientist selected by this society for scientific merits obtained and again it was within the field of hormone structure and action.
4. In 1987 I received a prize within biotechnology created by the Research Park at the University of Oslo, which at that time was called the Innovation Centre, University of Oslo.
5. Novum Lectures in Cellular and Molecular Biology, which was associated with a scientific prize. Invited by Professor Jan-Åke Gustafsson at Novum, Huddinge, The Karolinska Institute, Sweden, in 1991. This was given based on my research with human parathyroid hormone in relation to its first cloning, expression and studies of actions.
6. Lectures at Harvard School of Public Health in Cellular and Molecular Biology in 1995, regarding cloning of hormone genes and their characterizations. Invited by Professor A.H. Tashjian Jr. at the Department of Molecular and Cellular Toxicology, Harvard School of Public Health and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, USA.
7. Given a 3 years economical "Group Research Support of 0.8 M NOK per year from 1997" after national and international project evaluations.
8. Scientific prize (Abstract award) 1997, at the Endocrine Society "Cloning and Organization of the human TRH-receptor Gene".
9. Norwegian Endocrine Society Prize (S.Reppe) for "Sox4 – a PTH regulated transcription factor in bone".
10. Endocrine Society 1998: Poster Award: Cloning and regulation of the thyroliberin receptor gene.
11. Norwegian Endocrine Society prize 1998: Hormone regulated bone remodelling.
12. American Society for Bone and Mineral Research (ASBMR); 1999 Best Poster Award: The Transcription factor Sox-4 is expressed in developing cartilage and bone cells.

Consulting appointments:

1. Senior honorary consultant for NPS Biotechnology, Salt Lake City, Colorado, USA.
2. Consultant for Karolinska Institute, Stockholm, Sweden.

Referee activity:

I am or have been working as referee for the following international journals:

Endocrinology, J. Expl. Cell Res., Acta Physiol. Scand. (Kbh.), Eur. J. Endocrinol. (Acta Endocrinol. Scand. (Kbh.)), Eur. J. Clin. Invest., Hormone Research, Acta Obstet. Gynecol. Scand., Journal of Endocrinological Investigation, Eur. J. Biochem., Experimental Cell Research, J. Biol. Chem.

Guidance for the academic doctor degree: Twenty three and 5 ongoing.

Supervision of postgraduate candidates: Presently three.

Supervision of students' main degrees: Nineteen.

Guest research workers from abroad: In my group we have had research visitors for periods of one to three years from Polen, Bulgaria, Sweden, Tyskland, Denmark, Iceland, India, Israel and USA.

Member of committees for the academic doctor degree in Norway and abroad: 15.

Member of advisory international/national committees for evaluation of professor positions: 14.

Honorary Societies: Member of the Norwegian National Academy of Science and Letters

Professional memberships: Norwegian Society of Biochemistry, Norwegian Society of Physiology, Norwegian Society of Endocrinology, Endocrine Society (USA), American Society for Bone and Mineral Research (USA)

Medical Faculty Responsibilities:

1. An elected member of the Medical Faculty 1987-1990.
2. A member of the Research Council at the Medical Faculty 1987-1990.
3. Chairman of Postgraduate Courses for Ph.D. and Dr.med. students at the Medical Faculty 1986-1991.
4. Member of the Institute Group Committee for the Preclinical Sciences from 1989 and present.
5. Member of the Medical Faculty's council for evaluation of postgraduate applications from 1989-1993.
6. Committee member of the Medical Faculty's Scientific Instrument Board, 1996-.
7. Committee for Medical Research collaboration and interaction between University of Oslo and the National Hospital, 2000-.

National- and International Research Council Responsibilities:

1. Leader of Chemical Peptide Synthesis Core facility 1984-1989.

2. Chairman for the Biotechnology Committee as a representative for Norwegian Research Council in an inter research council body, 1986-1989.
3. Member of The Norwegian Research Council for Science and the Humanities (NAVF) Committee for Physiology and Pharmacology, 1986-1989.
4. Development and function as responsible leader of the nationwide core facility for peptide synthesis, 1988-1991.
5. Member of the Premedical Institute Group Committee for Preclinical Sciences from 1989-2003.
6. Member of the International Scientific Board of Novo-Nordisk Research Committee, 1989-2001.
7. Member of the CIBA Foundation Scientific Advisory Panel from 1995 elected as representative from Norway, 1989-present.
8. Chairman of the Research Council in the Norwegian Association for Osteoporosis, 1993-2003.
9. Leader of DNA Sequencing Core facility of the Institute of Basic Science, 1999-present.
10. *Consultant and peer reviewer within Wallenberg Consortium North Technology Platforms DNA; SNP (single nucleotide polymorphism) Technologies and the Platform for Proteomics on behalf of the Board of the Wallenberg Consortium North, Stockholm, Sweden, 2001-2004.*
11. *Coordinator for Marie Curie Training Sites Fellowship No MCFH-200-00040 : "Oslo Doctoral Training Site for Diagnosis and Therapy of Osteoporosis"—2001-2005.*
12. *Coordinator of EU 6.Program STREP contract no 502941, "Molecular mechanisms of bone homeostasis" (OSTEOGENE). Eight partners in 5 countries- 2003-2006*

Awards and fellowships:

1967, 3 months, I worked as a lecturer at the Department of Physiology, Medical School, Birmingham University, England.

1971 to July 1973 in receipt of Fogarty international post-doctoral fellowship at the Department of Pharmacology, Harvard School of Dental Medicine and Harvard Medical School.

1980, 4 months Visiting Professor at Institute of Genetics, BMC, Uppsala University, Sweden.

1995/96, 12 months Visiting Research Professor, The Scripps Research Institute, Dept. Mol. Biology, La Jolla, San Diego, USA.

1997 Awarded for 3 years the Norwegian Research Council's Science Prize for outstanding research.

2001 Promoted by EU to become Oslo Doctoral Training Site for Diagnosis & Therapy Of Osteoporosis, received by a group consisting of scientists from University of Oslo, IMBA and the National Hospital.

2002 Member of CNS Molecular Biology group (leader Ivar Walaas) appointed as a "Research Theme Priority" at the Medical Faculty, 2002- 2007

2004 OSTEOGENE (Molecular mechanisms of bone homeostasis) project given the highest priority and the only selected for presentation within Health Region East 2004.

Other professional activities:

1. Founder of the Norwegian Association for Osteogenesis Imperfecta 1978 (Norsk Forening for Osteoporosis Imperfecta) together with Mrs. L. Myhre.
2. Founder of the Norwegian Association for Osteoporosis (Norsk Osteoporoseforening) 1993, together with Norwegian Women Public Health Association (NKS).

Patents:

I. Two U.S. patents, U.S. Patent No. 5.010.010 and No. 5.420.242 are held with international extensions in Europe, Japan, Canada, and Australia. In addition, three Divisional Applications are submitted to the U.S. Patent Office and elsewhere. These patents and patent applications in the different countries are covering specific methods related to the production, purification and characterization of PTH in microorganisms for the use in treatment of osteoporosis.

II. Inventor in patent application from Scripps Research Institute on: Novel hypothalamic mRNAs, the corresponding peptides and their functions.

Publications: *More than 200 original articles published in internationally well reputed and refereed journals. Relevant articles are cited in relation to description of the research activities :*

A BRIEF DESCRIPTION OF THE MAIN RESEARCH PROJECTS AND RELEVANT REFERENCES

A. STUDIES OF HYPOTHALAMIC SPECIFIC mRNAs OBTAINED BY A NOVEL SENSITIVE SUBTRACTION HYBRIDIZATION PROCEDURE

The results so far from our refinement and usage of a powerful and highly sensitive novel subtractive nucleic acid hybridization method have been successful. The generated hypothalamic subtraction library appears to give a specific and comprehensive representation of mRNAs that are not present in other brain areas as hippocampus and cerebellum. We have so far described several novel peptides: hypocretin (the cause of Narcolepsy) and several very interesting peptides, e.g. novel CaM kinase (see list of references). Another CNS peptide is somatostatin-like, called cortistatin, structure similarity with somatostatin; P25 and Vat 1, two uniquely expressed peptides in distinct regions of the brain.

1. Gautvik, K.M., de Lecea, Luis, Gautvik, V.T., Danielson, P.E., Tranque, P., Dopazo, A., Bloom, F.E. and Sutcliffe, J.G. Overview of the most prevalent hypothalamus-specific mRNAs identified by directional tag PCR subtraction.

Proc. Natl. Acad. Sci. USA (PNAS) 93: 8733-8738, 1996.

2. de Lecea, L., Criado, J.R., Prospero-Carcia, O., Gautvik, K.M., Schweitzer, P., Danielson, P.E., Dunlop, C.L.M., Siggin G.R., Henriksen, S.J. and Sutcliffe, J.G. A cortical neuropeptide with neuronal depressant and sleep-modulating properties. Nature 381: 242-245, 1996.

3. de Lecea, L., Kilduff, T.S., Peyron, C., Gao, X-B., Foye, P.E., Danielson, P.E., Fukuhara, C., Battenberg, E.L.F., Gautvik, V.T., Bartlett II, F.S., Frankel, W.N., Van den Pol, A.N., Bloom, F.E., Gautvik, K.M. and Sutcliffe, J.G. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc. Natl. Acad. Sci. USA (PNAS) 95: 322-327, 1998.

B. ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR THE BONE CELL PHENOTYPE OBTAINED BY SUBTRACTION HYBRIDIZATION

By using the same novel subtractive hybridization procedure as employed and described above, we have generated a subtracted cDNA library using the osteosarcoma phenotype cDNA library as made from three different human osteosarcoma cells from which is subtracted the cDNA library obtained from normal human osteoblasts. The subtraction is performed by using cDNA from osteosarcoma cells minus RNA transcribed from the corresponding cDNA library of the normal osteoblast. These are experiments in progress and we are about to describe individual clones obtained from a subtracted library of about 400.000 independent colonies. The aim of this study is to identify those mRNAs which are overexpressed or lacking in the osteosarcoma phenotype and compile these results in order to have a greater understanding regarding how a normal cell is transformed into this tumor type (Olstad et al. ,2003)

C. ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR PARATHYROID HORMONE GENE ACTIVATION IN BONE CELLS ("Bone anabolic genes")

Parathyroid hormone is the most important physiological regulator of bone formation. This hormone therefore is assumed to represent an important drug in the prevention and especially treatment of postmenopausal osteoporosis. However, as a succession of our previous work regarding the studies of this hormone, we have continued to search for a complete overview of all gene products that parathyroid hormone is stimulating in bone cells in order to isolate the mRNAs and corresponding proteins which may be of central importance for the development of osteoporosis - or which may be called "the genes for osteoporosis". Again by using the same molecular subtraction method as described in Chapter II, C, we use this time parathyroid stimulated normal bone cells cDNA library minus RNA transcribed from generated libraries of normal bone cells. This work is almost completed in a highly successful manner. We have isolated more than 40 genes which are involved in PTH anabolic action in bone, and among those we are searching for the gene(s) causing postmenopausal osteoporosis.

D. As a complementation an to the activities described above, we have embarked on defining the bone phenotype in female and male osteoporosis within the context of the EU project OSTEOGENE (see above). About 100 patients and controls will have bone biopsies which will be prepared and analysed for their global gene expression and differences at the micro- and ultrastructural level. I am the coordinator of this activity including 5 countries and where Oslo university and three hospitals(Ullevål university hospital, the National Hospital and Lovisenberg hospital are working closely together. This is a direct consequence and follow up of previous research representing patient related basic and translational science aiming to solve the mechanisms of osteoporosis, the most common disease in women of 50 yrs of age.

THE MAIN RESEARCH ACTIVITIES DURING THE LAST 8 YEARS AND FUTURE SCIENTIFIC ENGAGEMENT:

I. PARATHYROID HORMONE (PTH) AND PARATHYROID HORMONE RELATED PROTEIN (PTHrP)

The aim for this work was to produce:

- i) Recombinant parathyroid hormone for structure activity studies in relation to bone cell activation.
- ii) Study intracellular processing and trafficking of these hormones and to compare signal sequence efficacy in different host expression systems.

We were the first in the world to clone and produce full-length human recombinant parathyroid hormone in mg quantities. For this work we developed gene constructs, vector modifications, fermentation technological improvements as well as complete methods for down-stream technology. The final product is PTH identical and more than 99% pure and has shown full chemical, biochemical and biological identity with the intact hormone. These results are written in the following articles that are printed.

We have also been as indicated by the list of references below, the first in the world to express secreted human parathyroid hormone in mammalian cells as well as a secretory milk product in transgenic mice. In addition, we have been the first to develop full-length PTH polypeptides with agonist and antagonist functions.

1.Høgset, A., Blingsmo, O.R., Gautvik, V.T., Sæther, O., Jacobsen, P.B., Gordeladze, J.O., Alestrøm, P. and Gautvik, K.M. Expression of human parathyroid hormone in *Escherichia coli*. BBRC 166: 50-60, 1990.

2.Gabrielsen, O.S., Reppe, S., Sletten, K., Øyen, T.B., Sæther, O., Høgset, A., Blingsmo, O.R., Gautvik, V.T., Gordeladze, J.O., Alestrøm, P. and Gautvik, K.M. Expression and secretion of human parathyroid hormone in *Saccharomyces cerevisiae*. Gene 90(2): 255-262, 1990.

3.Høgset, A., Blingsmo, O.R., Sæther, O., Gautvik, V.T., Holmgren, E., Josephson, S., Gabrielsen, O.S., Gordeladze, J.O., Alestrøm, P. and Gautvik, K.M. Expression and characterization of a recombinant human parathyroid hormone secreted by *E.coli* employing the staphylococcal protein A promoter and signal sequence. J. Biol. Chem. 265: 7338-7344, 1990.

In this regard we have received acceptance for an international patent on gene constructions, plasmids, the process and the down-stream technology. In the further work we have by using in vitro mutagenesis, created full length parathyroid hormone agonist which has shown to be protease resistant and have interesting biological actions regarding mobilization of calcium from bone.

Both the intact hormone as well as the agonist will represent important medical drugs for use in diagnostics as well as represent a potential drug for treatment of various diseases.

4. Reppe, S., Olstad, O.K., Blingsmo, O.R., Gautvik, V.T., Sæther, O., Gabrielsen, O.S., Øyen, T.B., Gordeladze, J.O., Haflan, A.K., Tubb, R., Morrison, N., Tashjian, A.H. Jr., Alestrøm, P. and Gautvik, K.M. Successful cloning and production of human parathyroid hormone (hPTH) in yeast as a secretory product. ECB, 5th European Congress on Biotechnology, Copenhagen July 8-14, 1990. (Invited).

5. Reppe, S., Gabrielsen, O.S., Olstad, O.K., Morrison, N., Sæther, O., Blingsmo, O.R., Gautvik, V.T., Gordeladze, J.O., Haflan, A.K., Voelkel, E.F., Øyen, T.B., Tashjian A.H. Jr. and Gautvik, K.M. Production of recombinant human parathyroid hormone in yeast: Synthesis, purification, and biological characterization of a Lys-26Gln site directed mutant. J. Biol. Chem. 266: 14198-14201, 1991.

6.Forsberg, G., Brobjer, M., Holmgren, E., Bergdahl, K., Persson, P., Gautvik, K.M. and Hartmanis, M. Thrombin and H64A subtilisin cleavage of fusion proteins for preparation of human recombinant parathyroid hormone. J. Protein Chem. 10(5): 517-526. 1991.

7.Olstad, O.K., Reppe, S., Gabrielsen, O.S., Hartmanis, M., Blingsmo, O.R., Gautvik, V.T., Haflan, A.K., Christensen, T.B., Øyen, T.B. and Gautvik, K.M. Isolation and characterization of two biologically active O-glycosylated forms of human parathyroid hormone produced in

Saccharomyces cerevisiae. Identification of New Motif for O-glycosylation. Eur. J. Biochem. 205: 311-319, 1992.

8.Kareem, B.N., Rokkones, E., Høgset, A., Holmgren, E. and Gautvik, K.M. A method for the evaluation of the efficiency of signal sequences for secretion and correct N-terminal processing of human parathyroid hormone produced in *Escherichia coli*. Anal. Biochem. 204: 26-33, 1992.

Recently we have expressed the first known full length antagonist for hPTH, a long sought for molecule of considerable clinical interest. The compound has a binding KD which is 2-4 times less than the natural hormone, but shows a more than 100-fold reduced biological activity.

9.Rian, E., Jemtland, R., Olstad, O.K., Gordeladze, J.O. and Gautvik, K.M. Expression of biologically active human parathyroid hormone-related protein (1-141) in *Saccharomyces cerevisiae*. Eur. J. Biochem. 213: 641-648, 1993.

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11.Kareem, N.B., Rokkones, E., Høgset, A., Holmgren, E. and Gautvik, K.M. Translocation and processing of various human parathyroid peptides in *E.coli* are differentially affected by protein A signal sequence mutation. Eur. J. Biochem. 220: 893-900, 1994.

12.Rokkones, E., Kareem, B.N., Olstad, O.K., Høgset, A., Schenstrøm, K., Hansson, L. and Gautvik, K.M. Expression of human parathyroid hormone in mammalian cells, *Escherichia coli* and *Saccharomyces cerevisiae*. J. Biotechnol. 33: 293-306, 1994.

13.Olstad, O.K., Morrison, N.E., Jemtland, R., Jüppner, H., Segre, G.V. and Gautvik, K.M. Differences in binding affinities of human PTH(1-84) do not alter biological potency: A comparison between chemically synthesized hormone and recombinant forms. Peptides 15: 1261-1265, 1994.

14.Rokkones, E., Fromm, S.H., Kareem, N.B., Olstad, O.K., Høgset, A., Klungeland, H., Iversen, J., Bjørø, K. and Gautvik, K.M. Human parathyroid hormone as a secretory peptide in milk of transgenic mice. J. Cell. Biochem. 59: 168-176 1995.

15.Olstad, O.K., Jemtland, R., Loseth, O.P., Brighurst, F.R. and Gautvik, K.M. Expression and characterization of a recombinant human parathyroid hormone partial agonist with antagonistic properties: Gly-hPTH(-1+84). Peptides 16: 1031-1037, 1995.

16.Mathavan, S., Gautvik, V.T., Rokkones, E., Olstad, O.K., Kareem, B.N., Maeda, S. and Gautvik, K.M. High level production of human parathyroid hormone in *Bombyx mori* larvae and BmN cells using recombinant baculovirus. Gene 167: 33-39, 1995.

17.Olstad, O.K., Reppe, S., Løseth, O.P., Jemtland, R. and Gautvik, K.M. Binding and Cyclic AMP Stimulation by N-terminally Deleted Human PTHs (3-84 and 4-84) in a Homologous Ligand Receptor System. J. Bone & Mineral Res. 12: 1348-1357, 1997.

18.Jemtland, R., Lee, K. and Segre, G.V. Heterogeneity among cells that express osteoclast-associated genes in developing bone. Endocrinology 139(1): 340-349, 1998.

19.Jemtland, R., Rian, E., Olstad, O.K., Haug, E., Bruland, O.S., Bucht, E. and Gautvik KM. Two human osteoblast-like osteosarcoma cell line show distinct expression and differential regulation of parathyroid hormone-related protein. J. Bone Miner. Res. 14(6): 904-14, 1999.

20.Reppe, S., Rian, E., Jemtland, R., Olstad, O.K., Gautvik, V. and Gautvik, K.M. SOX-4 mRNA is expressed in the embryonic growth plate and regulated via the PTH / PTHrP receptor in osteoblast-like cells. J.Bone Min.Res., 15,2402-2412, 2001.

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can prove to be the most versatile.

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Determining Sequencer Sensitivity

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Exh B

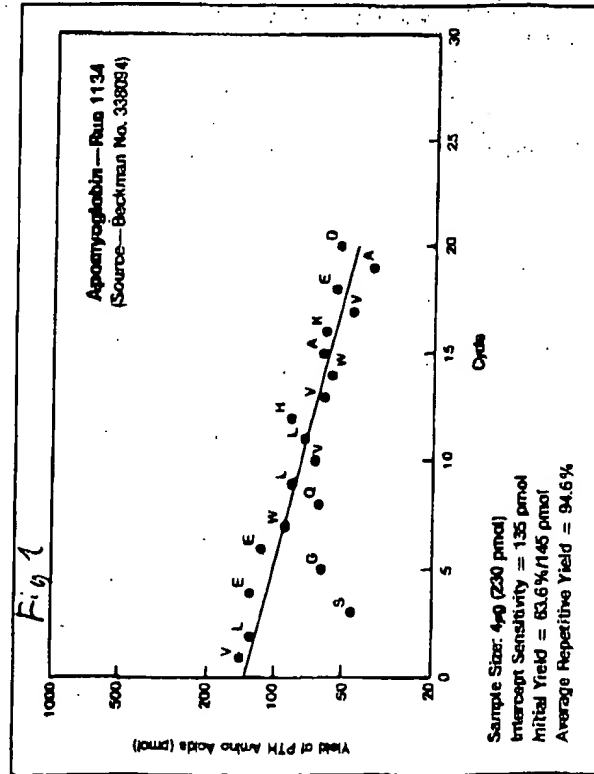
To determine and evaluate the sensitivity of a sequencer system, a number of different factors have been used over the years. Among the determinants have been the intercept of the Y-axis of the repetitive yield plot and the initial yield of the first PTH-amino acid recovered.

Also important to know is the initial amount and full description of the sample, as well as having actual PTH chromatograms available to evaluate the amount of background noise relative to the peak being identified.

To provide a better basis for comparing data, specific determinants of sensitivity are suggested below. Included are definitions and, where appropriate, examples of each of the suggested determinants.

SAMPLE SIZE The amount of sample, by weight and/or by picomoles or nanomoles, actually loaded into the reaction vessel.

AVERAGE REPETITIVE YIELD Least squares linear regression plot of individual amino acids quantitatively recovered at each sequenced cycle.

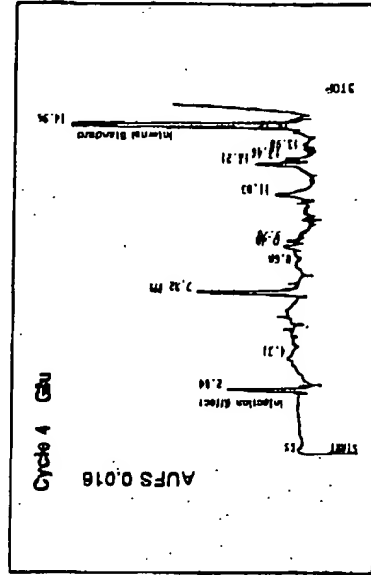


Average Repetitive Yield Plot

INITIAL YIELD Recovery of PTH-amino acid in the first cycle, in both % yield and picomole (or nanomole) amounts.

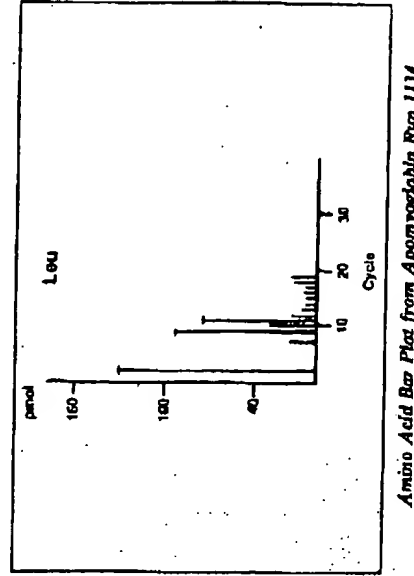
INTERCEPT SENSITIVITY Extension of the average repetitive yield plot to the Y-intercept: the value at that point

PTH CHROMATOGRAMS Actual HPLC chromatograms showing the separation and quantitation of the PTH-amino acids. Background subtraction or "data enhancement" (if any) should be indicated.



PTH-Amino Acid Chromatogram from Apomorphin Run 1134

AMINO ACID BAR PLOT Recoveries of an individual amino acid over the total number of cycles run; indication of background levels.



The sensitivity of a sequencer system is dependent on a number of factors:

- Sample composition
- Quality of reagents and chemicals
- Operator expertise
- Techniques of PTH amino acid analysis
- Quality of the sequencer system

The usefulness of a sequencer system may also be determined by its ability to maintain sensitivity over a wide range of loaded sample sizes.

Protein Sequence Determination

B. A. C.

A Sourcebook of Methods and Techniques

Edited by Saul B. Needleman

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Chapter 9

Analysis of Amino Acid Phenylthiohydantoins by Gas Chromatography and High Performance Liquid Chromatography

JOHN J. PISANO

I. General Methods for PTH Identification

Amino acid phenylthiohydantoins (PTHs) are formed in Edman's now classic technique for determining the primary structure of peptides and proteins. Introduced over 20 years ago, it is still the most effective and widely used method for sequence analysis. While X-ray crystallographic and mass spectrometric approaches remain attractive because they do not involve tedious step-wise analyses, their utility is restricted by requirements inherent in the methods. Thus, for the X-ray technique, suitable crystals and adequate stability during X-ray bombardment are unattainable for many proteins and peptides. Even with good crystals, unambiguous distinction of all amino acids is often impossible with obtainable data. Limited volatility is the main deterrent to the wider use of mass spectrometry in peptide analysis. Greater use of the method will follow improvements in chemical techniques for converting peptides to suitable derivatives, but it is unlikely that it will ever be possible to analyze peptides containing more than about 15 residues.

Analysis of PTHs formed in the step-wise Edman degradation was first achieved by paper [146, 1165] and thin-layer chromatographic methods [222, 320]. Although thin-layer chromatography remains an important method of analysis, many investigators have sought alternative methods because the procedure is tedious, difficult to quantitate and limited in resolving power. Identification of some derivatives requires as many as four different solvent systems.

Several valuable indirect procedures have been developed to overcome these difficulties including hydrolysis of the PTHs [9, 1181], the subtractive-Edman [549, 720] and the dansyl-Edman methods [438, 439]. All three procedures circumvent the need to determine the amino acid PTH directly. In the hydrolytic method, the PTH [9] or thiazolinone [1181] is hydrolyzed under carefully controlled conditions and the recovered amino acid is identified and quantitated by conventional means. A major advantage of this method is the lack of interference from contaminants present in the sample or arising from the buffer and solvents. Larger aliquots of the PTH sample may be taken for analysis than with direct methods of PTH analysis which are subject to interference by gross contaminants (Ettore Appella, personal communication). Possible disadvantages include the labor and expense of amino acid

analysis, the need for two hydrolytic methods and the possible uncertainty of asparagine and glutamine determinations because they are based on ammonia production [1181].

In the subtractive-Edman method [549, 720] the sequence is deduced by amino acid analysis of an aliquot of the shortened peptide after the cleavage and conversion steps of the Edman method. While this approach will undoubtedly continue to be highly useful it has certain drawbacks. It is limited to small peptides where the loss of a single residue may be determined with confidence. Loss of sample at each step, accumulation of blocked peptides which obscure the analysis, expensive and time-consuming amino acid analysis, and the need to use other methods to identify asparagine, glutamine and tryptophan residues are other limitations of the subtractive method.

In the more sensitive dansyl-Edman procedure, an aliquot of the shortened peptide is taken for identification of the new N-terminal amino acid using the dansyl technique [438, 439]. This valuable method has not worked well with proteins which are insoluble in the sodium bicarbonate-acetone buffer or have sterically hindered N-terminal amino acids. Other limitations include the need for additional methods to determine asparagine, glutamine and tryptophan residues and the difficulty in quantitating dansyl amino acids.

The ideal method for sequence analysis is one which would be applicable to proteins or large fragments and could be automated. Neither the subtractive-Edman nor the dansyl-Edman indirect methods are suited for this purpose.

Direct analysis of the PTHs formed at each step of the Edman procedure is, in principle, the most logical approach in sequencing. A further stimulus to the development of suitable methods for direct PTH analysis was the invention of the protein sequenator for automated Edman degradation [317]. In an attempt to overcome the limitations of the TLC and hydrolytic methods for PTH analysis cited above, gas chromatographic and more recently high performance liquid chromatographic methods [1427] have been developed. Both procedures offer high speed sensitivity, specificity and ease of quantitation.

II. Gas Chromatography

The first demonstration that most amino acid PTHs could be successfully analyzed by gas chromatography (GC) also revealed difficulties with the seryl, threonyl, asparaginy, glutaminy, lysyl and arginyl derivatives [985]. These difficulties were overcome [453, 501, 980, 981, 982, 984] by the use of new thermally stable and more polar polysiloxane liquid phases and the powerful silylating reagents, N,O-bis-(trimethylsilyl)acetamide (BSA) [703] or N,N-bis(trimethylsilyl)trifluoroacetamide (BSTFA) [1210] which readily convert the less volatile and unstable PTHs to trimethylsilyl derivatives having excellent chromatographic properties. The very polar argininy PTH does not form a stable silyl derivative and cannot be analyzed by GC.

A. Equipment

Several manufacturers offer suitable instruments. However, glass columns and on-column injection are recommended because destruction of PTHs occurs in metal

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injection ports and columns. A hydrogen flame detector is also recommended because of its high sensitivity, thermal stability, wide linear dynamic range and ruggedness. To accommodate the large number of samples produced in the automated Edman degradation, the capacity for the analysis of two samples simultaneously is recommended. This may be accomplished most economically with a double column oven, two hydrogen flame detectors, electrometers and recorders and one temperature programmer. With this instrument package and simultaneous injection, one has the capacity of two chromatographs but saves the cost of a separate column oven and temperature programmer. Busy laboratories may employ two such instruments and take full advantage of the many complementary columns which can be employed.

B. Materials

The support, Chromosorb W, 100 to 120 mesh, stationary phases DC-560 (or the equivalent SP400), OV-210, OV-225, silylating reagents BSA, BSTFA, and dichlorodimethylsilane may be purchased from various supply houses (e.g. Supelco, Inc., Bellefonte, Pa.; Pierce Chemical Co., Rockford, Ill.).

C. Preparation of the Support

The preparation of the support [584] has been modified to include a Na_2CO_3 prewash which slightly improves column efficiency [981]. Chromosorb W, 50 g, is mixed with 500 ml of 0.5 M Na_2CO_3 in a 1 l beaker. After standing overnight fine particles are removed by several decantations using distilled water. Concentrated HCl, 500 ml, is added to the almost neutral support and after 16 to 24 hrs standing with occasional swirling, the fine particles are again removed by several decantations using distilled water. The support is dried at 140° and, while still warm, a 25 g portion is transferred to a 1 l flask. Approximately 200 ml of a 5% solution (v/v) of dichlorodimethylsilane in toluene is added and the mixture degassed by the use of an aspirator. The flask is gently swirled 2 or 3 times during degassing and the 10 to 15 min reaction period. The excess reagent is decanted and the support is rinsed 3 times with anhydrous (reagent grade) toluene. Since the support may contain bound reactive silyl chlorides it should be protected from atmospheric moisture. This is conveniently achieved by keeping the support wet with toluene and carrying out the washings without interruption. Decatination is effected by the addition of 300 ml of anhydrous methanol. After standing 10 to 15 min, the methanol is decanted and the support is rinsed with anhydrous methanol until the rinses are clear. The mixture is then filtered using a coarse sintered-glass funnel, the support is rinsed with acetone while in the funnel, air-dried and finally dried in an oven at 140° .

Removal of the fine particles produced during the above procedures is recommended for the preparation of efficient columns. After the acid and base washes it may be necessary to decant 25 times with distilled water. Chromosorb W is a fragile and support is easily crushed. Since crushed material contains newly exposed active sites, it should not be mixed with intact support.

The filtration technique is used to coat the support with stationary phase [584]. The amount of stationary phase used is expressed as its percentage (w/v) in solvent.

recommended because of its ruggedness. The automated Edman simultaneously is recommended for double column oven, and one temperature injection, one has the column oven and such instruments and which can be employed.

uses DC-560 (or the STFA), and dichloro- (e.g. Supelco, Inc.,

include a Na_2CO_3 , mosorb W, 50 g, is added overnight fine water. Concentrated 5 to 24 hrs standing several decantations still warm, a 25 g % solution (v/v) of added by the use of using and the 10 to e support is rinsed support may contain atmospheric moisture. Chloroform and carrying by the addition of methanol is decanted nses are clear. The c support is rinsed oven at 140°.

cedures is recommended and base washes it mosorb W is a fragile wly exposed active

ionary phase [584]. ge (w/v) in solvent.

Thus a 10% DC-560 packing is prepared with 10 g of DC-560 made up to 100 ml with acetone. Usually, 75 ml of solution is added to 5 g of support in a 125 ml filter flask. The mixture is degassed by gentle swirling while under reduced pressure (aspirator), filtered on a 150 ml sintered glass funnel until apparently dry, transferred to a dish and thoroughly dried at 140°. The volume of coating solution is not critical but should be sufficient to allow transfer of the support (with swirling) to the sintered glass funnel.

Of the numerous stationary phases tested, none is capable of separating all the PTHs. However, two phases, DC-560 (or SP400) and XE-60 (or the similar OV-225) are complementary; PTHs unresolved with one phase are resolved with the other.

A superior single column is obtained with a blend of phases referred to as CFC (18) consisting of equal volumes of acetone solutions of 5.5% SP400, 4% OV-210, and 0.5% OV-225.

D. Preparation of Columns and Chromatographic Conditions

Glass columns approximately 4 feet \times 2 mm i.d. and glass wool plugs are silylated like the support with dichlorodimethylsilane in toluene. Columns are filled with the reagent and the glass wool is soaked in it for about 15 min. When filling the columns, they are gently tapped to promote even packing. A vibrator should not be used as it may damage the support or cause it to pack too tightly giving prohibitively slow flow rates.

Columns are conditioned using an initial temperature of approximately 50° and a helium flow rate of 150 ml/min. After about 30 min the temperature is raised at the rate of 0.5°/min until it reaches 290°, where it is held for at least 16 hrs or until the baseline rise is less than about 10% full scale when the temperature is increased from 170 to 290°. Helium is superior to nitrogen and argon carrier gases, giving better resolution and a wider latitude of flow rates without affecting efficiency [981]. Some laboratories may prefer nitrogen because it costs less and still gives satisfactory results. Ultrapure gasses have been used throughout; lower grades of purity have not been tested.

Analyses are usually performed with the injector temperature at 250 to 270°. At lower temperature, e.g., 200°, volatilization of asparaginyl, glutaminyl, tyrosyl, lysyl, histidyl and tryptophanyl PTHs may be incomplete. Temperatures higher than 270° may cause decomposition of glutaminyl, lysyl, and histidyl PTHs. At 300° most PTHs decompose. The hydrogen flame detector bath is held at 300°. Most analyses are performed at column temperatures ranging from 165° to 290°. The optimum temperature should be determined by the investigator. Electrometer controls are usually set so that a full scale deflection corresponds to about 3×10^{-10} A with a 5 mV recorder.

It is not uncommon for the first few samples to tail on a new column. After a few analyses the columns stabilize and may be used for months or even years if not abused by the use of excessive temperatures or dirty samples. The unsuitability of a column is determined by the increase in baseline rise during temperature programming and the decrease in resolution or yield, or the disappearance of peaks. Contaminated columns are usually discarded, but removal of the top inch of packing will occasionally improve the column.

E. Standard Solutions

A suitable solvent for all the PTHs has not been found. Ethyl acetate and ethylene dichloride, used by Edman, will not dissolve sufficient crystalline asparaginyl, glutaminyl and histidinyl PTHs to give the convenient concentration of 1 mg/ml. Methanol is a good solvent but it cannot be used when the standards are silylated (see below). Although reagent grade acetonitrile, N,N-dimethylformamide and pyridine are also good, PTHs are unstable in these solvents. In ethyl acetate, ethylene dichloride and methanol, however, they are stable for months when stored in the dark below 5°. Derivatives obtained from a degradation are much less stable than crystalline standards in any solvent. Upon storage overnight some derivatives (especially seryl PTH) may be completely destroyed, presumably due to contaminants in the sample.

F. Silylation of Phenylthiohydantoins

Because amino acid PTHs differ greatly in their chemical and chromatographic properties, it is useful to divide them into three groups (Table 9-1). Group I derivatives are most volatile and generally give symmetrical peaks. Group II derivatives are least volatile and, with the exception of tryptophan, show the greatest tendency to adsorb to the column packings giving tailing peaks and low responses. Group III derivatives include those (aspartic, glutamic and cysteic acids) which must be converted to the volatile silyl derivatives before analysis and others which when silylated have significantly better chromatographic properties [453, 501, 980, 981, 982, 984]. Silylation of Group I and II derivatives can provide useful confirmatory data. One laboratory [530] prefers to silylate all PTHs routinely and thereby reduce the number of analyses necessary to identify an unknown. However, chromatograms are often much more complex following silylation and occasionally members of Groups I and II may be missed if not analyzed before silylation.

Table 9-1. Grouping of amino acid PTH derivatives according to gas chromatographic behavior

Group I	Group II	Group III
Alanine	Asparagine	Aspartic acid
Glycine	Glutamine	S-Carboxymethylcysteine
Valine	Tyrosine	Cysteic acid
Leucine	Histidine	Glutamic acid
Isoleucine	Tryptophan	Lysine
Methionine		Serine
Proline		Threonine
Phenylalanine		

Group I amino acids are most volatile and generally give symmetrical peaks. Members of Group II are least volatile and with exception of tryptophan show the greatest tendency to adsorb to the column packing giving tailing peaks and low responses. Group III derivatives include those which must be silylated before analysis (aspartic, glutamic, and cysteic acids) and others which, when silylated, have significantly better chromatographic properties.

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INST. MED. BIOKJEMI +47 22851058

NO. 4231 P. 1

Environ. Health Perspect. Determination

Application of Sequenator Analyses to the Study of Proteins[†]

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and Kenneth A. Walsh*

ABSTRACT: The method of sequenator analysis described by Edman and Begg (Edman, P., and Begg, G. (1967), *Eur. J. Biochem.* 1, 80) has been modified and applied to proteins and protein fragments. Significant modifications include the replacement of Quadrol by a volatile buffer (dimethylbenzylamine), the introduction of thiols to stabilize the reaction products, and the identification of the reaction products as silylated phenylthiohydantoin by automated gas-liquid chromatography. With these and other modifications, 30–50

amino acid residues can be identified and recovered with a repetitive yield of approximately 96%. This modified method has been tested on thermolysin and its cyanogen bromide fragments and found to be reliable in determining amino acid sequences. It has also been applied to porcine trypsin and found to be of use in determining purity, allotypic variants, and internal peptide-bond cleavage. In addition, the chemical nature of protein subunits can be identified by this method.

The sequential degradation of peptides by the method of Edman (1956) is an important procedure for the determination of amino acid sequences of proteins. The method combines the specificity of end-group analysis with the advantages of a cyclic stepwise process and normally yields 7–15 unambiguous degradations. In 1967, Edman and Begg automated the process by designing an instrument called the "sequenator" and demonstrated its successful application to the identification of 60 amino-terminal residues of apomyoglobin. Since then, other sequenators have been constructed, built on the principles of Edman and Begg. According to published accounts, these instruments are capable of 20–50 consecutive degradations (*e.g.*, Niall and Edman, 1967; Morgan and Henschen, 1969; Rochat *et al.*, 1970; Hood *et al.*, 1970; Niall *et al.*, 1970; Brewer and Ronan, 1970; Niall *et al.*, 1971; Reeck *et al.*, 1971; Hermodson *et al.*, 1971; Glenner *et al.*, 1971; Jaton *et al.*, 1971; Smithies *et al.*, 1971; Titani *et al.*, 1972a).

The capability of the sequenator to determine long amino acid sequences has altered the general strategy of sequence analysis. Instead of fragmenting the protein into a large number of short peptides whose sequences can be determined by manual Edman degradations and by digestion with carboxypeptidases, the protein is cleaved into a small number of large fragments, usually by chemical procedures (*e.g.*, cyanogen bromide, hydroxylamine), and the separated fragments are directly subjected to automated sequence analysis. Only those segments which cannot be reached by the sequenator are subsequently analyzed by the classical procedures.

Sequenator analysis has also been effective for screening proteins for homology, simply by applying the sequential analysis to the amino-terminal region of the protein or to other regions adjacent to existing or newly created α -amino groups. Such initiation points for consecutive degradations can be established by chemical reactions or by limited enzymatic proteolysis.

Because of its sensitivity and the small amount of protein required for but a few turns, sequenator analysis is a rapid and

accurate test for protein purity and, *inter alia*, for determining the number of polypeptide chains in a pure oligomeric protein. The method also has proved useful in following the changes in covalent structure attending the activation of various pancreatic zymogens (Hermodson *et al.*, 1971; Pétra *et al.*, 1971).

We have applied the automated technique to proteins and protein fragments. Several changes in methodology developed in the course of this work have improved the reliability of the analysis and in most cases have enabled 30–50 sequential degradations. The direct identification of the reaction products as phenylthiohydantoinyl amino acids¹ by automated gas-liquid chromatography has proved particularly useful. Other methodological refinements include the replacement of Quadrol by dimethylbenzylamine in the coupling buffer and the use of thiols to stabilize the reaction products.

Because of the rapidly developing interest in the use of sequenators for sequence analysis of proteins, we wish to describe in this communication our present methodology and its application to several kinds of problems in protein chemistry.

Experimental Procedure

Reagents and Solvents. Phenyl isothiocyanate and heptafluorobutyric acid were prepared according to the method of Edman and Begg (1967).

Heptane, benzene, chlorobutane, and ethyl acetate were the "glass-distilled" grade of Burdick and Jackson Laboratories, Inc. 1-Propanol was purchased as the "Sequenal" grade from Pierce Chemical Co. These solvents were used without further purification.

N,N-Dimethylbenzylamine (Baker) was purified by treatment for 30 min with sodium borohydride (1 g/100 ml of DMBA) followed by four washes with distilled water. The reagent was then dried over CaSO₄ and vacuum distilled through a 40-cm Vigreux column from phthalic anhydride (5 g/100 ml of DMBA) under aspirator vacuum. The constant

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¹ The following abbreviations are used: DMBA, *N,N*-dimethylbenzylamine; Quadrol, *N,N,N',N'*-tetrakis(2-hydroxypropyl)ethylene-diamine; PTH, phenylthiohydantoin; DTE, 1,4-dithioerythritol.

TABLE I: Programs for Use with DMBA Buffer.^a

Step	Program Time (sec)		Cup Speed (rpm)
	General	Abbreviated	
Heptafluorobutyric acid delivery (reagent 3)	30	30	1200
Cleavage	200	200	1200
Restricted vacuum	30	30	1200
Rough vacuum	30	20	1200
Fine vacuum, N ₂ flushing	20	20	1200
Chlorobutane extraction (solvent 3)	150 (5 ml)	100 (4 ml)	1800
Restricted vacuum	100	80	1800
Rough vacuum	20	10	1800
Fine vacuum, N ₂ flushing	800	300	1800
Phenyl isothiocyanate delivery (reagent 1)	7	7	1800
Restricted vacuum	30	30	1800
N ₂ blow to atmosphere	30	30	1800
Buffer delivery (reagent 2)	20	20	1200
Coupling reaction	1800	1800	1200
Restricted vacuum	400	300	1200
Rough vacuum	40	40	1200
Fine vacuum, N ₂ flushing	50	100	1200
Benzene extraction (solvent 1)	300	300	1800
Restricted vacuum	100	80	1800
Rough vacuum	20	10	1800
Fine vacuum, N ₂ flushing	1200	600	1800

^a Bottle venting and pressuring and some N₂ blowing steps are not shown. Total time per cycle (including all steps) is approximately 95 min for the general program and 56 min for the abbreviated program. The complete program (39 steps) can be obtained from this laboratory on request.

boiling fraction was then redistilled through a 40-cm Vigreux column under vacuum (approximately 1 mm).²

The following reagents and solvents were used in the operation of the sequenator (Table I).

REAGENT 1 is 5% (v/v) phenyl isothiocyanate in heptane.

REAGENT 2, the buffer, is a mixture of 12 ml of DMBA, 41 ml of propanol, and 48 ml of distilled water, titrated to pH 9.4 with reagent grade glacial acetic acid. This buffer is approximately 0.8 M in DMBA. Since this concentration is very close to the limit of solubility of DMBA in the mixture, the solution should be clarified, if necessary, by dropwise addition of propanol.

REAGENT 3 AND SOLVENT 1 are heptafluorobutyric acid and benzene, respectively.

SOLVENT 3 is 0.1% (v/v) fresh reagent grade ethanethiol in chlorobutane. When placing this reagent in the instrument the reagent is flushed with nitrogen for only 2 min. Longer flushing removes ethanethiol.

Sequenator. The sequenator used in these experiments was a Beckman Sequencer (Model 890A) designed to adapt the

principles described by Edman and Begg (1967). A film of protein on the wall of a spinning cup is coupled in a nitrogen atmosphere with phenyl isothiocyanate in an appropriate buffer. Non-protein components are removed by a "rough" vacuum followed by a "fine" vacuum and finally by extraction with organic solvents. The dried protein film is then exposed to anhydrous heptafluorobutyric acid and the amino-terminal residue extracted in chlorobutane as a mixture of phenylthiohydantoin and thiazolinone. At this stage, one cycle of the Edman degradation is complete and a new cycle of the coupling and acid cleavage is begun. The chlorobutane extracts are separately treated to convert thiazolinone to thiohydantoin which is then identified as outlined below.

The particular instrument used was modified by insertion of a solenoid valve and a needle valve into the nitrogen line leading to the reaction chamber to introduce a regulated flow of nitrogen through the reaction chamber during the "fine" vacuum steps (see Table I). Thus the reaction chamber is purged with nitrogen during the evacuation, and the amount of condensation in the reaction chamber during operation with volatile buffers is greatly reduced. The N₂ flushing system is an optional addition to the Sequencer available from Beckman Instruments, Inc.

Programs. The standard programs of operation are shown in Table I. The general program is used when more than ten cycles are desired. The abbreviated program is faster and is satisfactory for ten cycles or less. The abbreviated program is sufficient for estimation of protein purity, for preliminary examination for internal breaks in a protein chain, or for amino-terminal analyses. Its usefulness is limited by the shortened drying periods which cause accumulation of condensates. Both programs start with a cleavage step so that the protein is always in the "coupled" state when the instrument is shut down automatically.

A cup speed of 1200 rpm was employed throughout the coupling and cleavage reactions. At the end of the delivery to the cup, the level of the heptafluorobutyric acid was 2–3 mm below the initial level of the buffer. This adjustment allowed for the partial evaporation of the buffer film during the coupling period and for the tendency of the acid to saturate the protein by capillary action. As a result the protein was completely covered during both the cleavage and coupling steps. Single cleavage was found to be adequate.

The temperature in the heated housing around the cup was 57°, measured with a calibrated thermometer lying on the metal base plate of the cup assembly.

Sequenator Maintenance. With proper programming and nitrogen flushing, there was little condensation on either the outer wall of the cup or the surrounding glass cylinder. However, some condensation inevitably occurred in the metal well housing the drive bearings below the cup. Hence the cup bearing assembly was removed after every extended run or after every 2 days and cleaned thoroughly with ethanol.

The use of a volatile buffer increased the accumulation of foreign material in the vacuum pump oil. With continuous operation of the sequenator, the oil in both pumps was replaced at least three times a week.

All glass test tubes in contact with sequenator products were cleaned in a hot solution of nitric and sulfuric acids (2:1, v/v), then rinsed exhaustively with distilled water, and dried.

Analysis of Sequenator Products. In order to avoid removal of the volatile thiol, the chlorobutane solutions of sequenator products were not automatically dried in the sequenator. Instead, the samples from 1 day's operation were dried quickly at approximately 60° under a stream of purified nitrogen and

² DMBA marketed as Sequenal grade by Pierce Chemical Co. is suitable for use without further purification.

immediately dissolved in 0.2 ml of 1 N HCl containing ethanethiol (1%, v/v). After 10-min standing at 80° to complete the cyclization of the phenylthiohydantoins (Edman and Begg, 1967), each solution was extracted with ethyl acetate. The ethyl acetate phase and the aqueous phase were separately examined for phenylthiohydantoins.

The ethyl acetate extracts were dried under nitrogen, silylated by adding 25–50 μ l of *N,O*-bis(trimethylsilyl)acetamide (1-ml ampoules from Pierce Chemical Co., stored at 4°), agitated in a Vortex mixer, and heated to 60° for 10 min.

The silylated products were identified by gas chromatographic analysis using an adaptation of the procedure of Pisano *et al.* (1972). A silylated glass column (2 mm i.d. \times 4 ft) was used. It contained "10%" SP-400 (Supelco Inc.) on deactivated acid-washed Chromosorb W. The sample (2–5 μ l) contained 5–40 nmoles of the PTH-amino acid. The gas flow was 140 cm³/min of helium and the instrument was programmed for temperatures ranging from approximately 190 to 290°. Programmatic adjustment of temperature was either linear or stepwise, depending on the capability of the instrument used. For optimal separation of silylated standard PTH-amino acids, minor adjustments in the temperature program were made for each newly packed column. Analyses were performed with either a Beckman GC-5 or a Hewlett-Packard 7620A instrument¹ equipped with flame ionization detectors. The yield of a PTH-amino acid was estimated by comparing the area under its peak on a gas chromatogram to that of a standard. Figure 1 shows the relative retention times of the silylated PTH-amino acids for a typical SP-400 column.

The large variations in polarity, volatility, and chemical stability among the PTH-amino acids make it necessary to sacrifice some desirable features of analysis in order to optimize others. A relatively polar stationary phase (e.g., SP-400) is necessary in order to separate the phenylthiohydantoins of alanine, δ -serine, valine, glycine, leucine, and isoleucine, but this impairs the quantitative chromatography of the polar and high-boiling PTH derivatives. A less polar stationary phase such as XE-60 gives better quantitation of the polar derivatives but for general sequencing work the added precision does not justify the additional time and effort of operating and servicing the columns required for this purpose.

On SP-400 the silylated PTH derivatives of alanine, glycine, valine, leucine, isoleucine, methionine, phenylalanine, proline, and tyrosine chromatograph very well and are easily quantitated. PTH-tryptophan is slightly more susceptible to destruction, especially by oxidation, and therefore is less easily quantitated. PTH-serine and PTH-threonine are exceedingly labile and any attempt to quantitate these residues appears futile. PTH-lysine, -asparagine, and -glutamine are polar and chromatograph poorly on SP-400; the peaks observed are symmetrical and discrete but the detector response does not correspond to their molecular weights. Since areas under the peaks of these compounds are not proportional to the amount of compound injected, quantitation is difficult. The sequenator products of aspartic acid and glutamic acid do not extract well from the cup (Edman and Begg, 1967) and hence their quantitation is not meaningful. PTH-S-carboxymethyl-

cysteine is degraded in the inlet port of the gas chromatograph and only PTH- δ -serine is observed. S-Methylcysteine appears to be the only cysteine derivative which can be quantitated by gas chromatography.

During repeated degradations, cumulative nonspecific cleavage of the protein unavoidably occurs. Consequently the background of PTH-amino acids gradually increases in amount with succeeding cycles. Thus in the later cycles, residues are identified by observing which peak increases relative to the background. Since the degradation is inevitably incomplete (approximately 96% recovery per cycle), the same residue will also be slightly above background in the subsequent cycle (termed "overlap").

Identification of residues that are labile or chromatograph poorly becomes difficult when the background and overlap are high and the overall yield of the residue is low. Serine, for instance, may be impossible to identify in a late cycle whereas valine, leucine, and alanine may be clearly identifiable ten or more cycles later. Hence sequenator experiments may end with blanks in the sequence rather than with an abrupt termination of useful data.

Thin-layer chromatography has essentially the same problems of residue identification. It is, however, easier to differentiate the residue at a given cycle from background on a gas chromatogram since gas chromatography is semiquantitative and not dependent on a subjective evaluation of spot intensity.

Identification of Histidine, Arginine, and Pyridylethylcysteine. Three PTH-amino acids remain in the aqueous phase after acid cyclization. Of these, PTH-histidine and PTH-arginine can be detected by diazotized *p*-anisidine and phenanthrenequinone spot tests, respectively,⁴ whereas PTH-pyridylethylcysteine is identified by thin-layer chromatography. The aqueous solutions are dried at 60° in a stream of nitrogen, then dissolved in 0.02 ml of methanol. A series of 0.005-ml aliquots of each methanolic solution is dried as spots on a strip of chromatography paper for detection of PTH-histidine and separately for detection of PTH-arginine. It is particularly important that all the aqueous layers be spotted in sequence on the paper to clearly differentiate histidine and arginine residues from the rising background and from overlap of the preceding cycle.

The histidine test will detect 15 nmoles and is performed according to the method of Sanger and Tuppy (1951). It is important that diazotization of *p*-anisidine proceeds for only 3–5 min before spraying the paper.

The arginine test will detect 10 nmoles and is performed essentially according to the procedure of Yamada and Itano (1966). Phenanthrenequinone (5 mg) is dissolved in 25 ml of absolute ethanol. A 4-ml aliquot of this solution is swirled on a Vortex mixer while adding 1 ml of 25% NaOH. The paper is dipped in this solution and air-dried for 20 min. Fluorescence under uv light is taken as a positive indication of PTH-arginine.

PTH-pyridylethylcysteine is identified by thin-layer chromatography with ethyl acetate on fluorescent silica gel (F-254, Brinkmann). The *R_F* of PTH-pyridylethylcysteine is approximately 0.4 while PTH-histidine and PTH-arginine remain at the origin.

¹ Aliquots (6 μ l) can be removed from 15- μ l samples in the automated Hewlett-Packard instrument by the use of disposable polyethylene 0.4-ml microcentrifuge tube inserts (Arthur H. Thomas 2591-D15). These tube inserts are forced into moistened glass ampoules (Hewlett-Packard sample vials) and the protruding tops are removed with a razor blade. The silylated sample is added and the ampoule capped. A special circuit board available from Hewlett-Packard (Option 504) controls a rinse of the syringe after each sample injection.

⁴ If the water layers are dried and silylated, both PTH-histidine and PTH-arginine can be recognized by gas chromatography. The histidine derivative appears as a discrete peak just before silylated PTH-tyrosine; the arginine derivative appears as a series of broad peaks near PTH-glutamic acid, probably corresponding to pyrolysis products.

TABLE II: Sequenator Analysis of Thermolysin and Fragments.^a

Fragment	Method of Preparation	Residue No. ^b	Residues Placed by Sequenator	
			No. of Degradations	Residues Identified
Thermolysin		1-316	15	1-15
F _{III}	Cleavage with CNBr ^a	1-120	33	1-33
S-F-III-T _I	Tryptic cleavage of succinylated F _{III} ^b	48-90	34	48-81
F _I	Cleavage with CNBr ^a	121-205	48	121-168
F _I -T _I	Tryptic cleavage of F _I ^b	183-205	8	183-190
F _{II}	Cleavage with CNBr ^a	206-316	32	206-237
F _{II} -HA ₂	Cleavage of F _{II} with NH ₂ OH ^c	228-316	44	228-271

^a The preparation of thermolysin and its cyanogen bromide fragments has been described by Titani *et al.* (1972b). ^b See Titani *et al.* (1972a). ^c Titani *et al.* (1972a) using the method of Bornstein (1969).

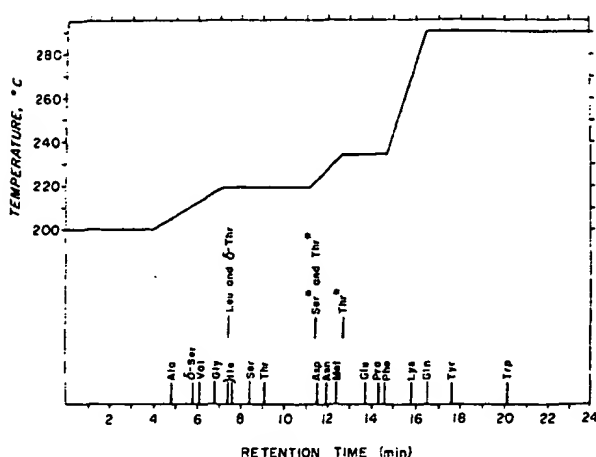


FIGURE 1: Retention times of silylated sequenator products during gas-liquid chromatography. The solid line traces the temperature program. Isoleucine residues yield a doublet at 7.4 and 7.6 min whereas leucine residues yield a single peak at 7.4 min (probably due to the formation of a diastereomeric pair during silylation of PTH-isoleucine). Thiols are included in extraction and cyclization procedures and influence the nature of the silylation products of PTH-proline, PTH-glycine, PTH-serine, and PTH-threonine (see text). With ethanethiol, serine residues yield both silylated PTH- δ -serine (the basis of their identification) and a presumed reaction product of silylated PTH- δ -serine and ethanethiol which cochromatographs with the aspartyl derivative (*). Threonine residues also yield multiple peaks: silylated PTH- δ -threonine which cochromatographs with the leucyl derivative (in low yield), silylated PTH-threonine, and a pair of thiol-dependent peaks (*) near the aspartyl and methionyl derivatives.

Preparation of Proteins for Sequenator Analysis. In general polypeptides are prepared for sequence analysis under conditions which minimize contamination or cleavage by proteases. Prior to analysis the polypeptides are tested for homogeneity by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate.

Cysteine and cystine are alkylated by an agent of choice.³ S- β -Pyridylethylation with 4-vinylpyridine (Friedman *et al.*, 1970) yields a suitable product. The derivative, pyridylethyl-

³ It was possible to avoid this procedure in some cases, e.g., bovine trypsin. Unmodified Cys₁ in trypsin yielded no extractable product but residues 8, 9, etc., were normally degraded.

cysteine, carries a positive charge in acid and, since this charge is not lost during treatment with phenyl isothiocyanate, extraction of the peptide in the chlorobutane is minimized.

The PTH derivative of S-carboxymethylcysteine appears to break down in the inlet port of the gas chromatograph since only PTH- δ -serine is observed after injection of PTH-S-carboxymethylcysteine. This renders the identification of both serine and cysteine ambiguous since PTH-serine is recognized primarily on the basis of the accompanying PTH- δ -serine peak. A simple way to resolve this ambiguity is to carboxymethylate the protein with [¹⁴C]iodoacetate (giving about 10,000 cpm/ μ mole of cysteine) and to scan aliquots of the chlorobutane extracts for ¹⁴C.

Aminoethylation with ethylenimine (Raftery and Cole, 1963) is not desirable for sequenator analysis since the new amino groups react with phenyl isothiocyanate adding to the hydrophobicity and lowering the solubility of the protein in the coupling buffer. In addition, the PTH derivative of S-aminoethylcysteine does not chromatograph on SP-400 and has the mobility of PTH-lysine on thin-layer chromatography.

S-Methylation with methyl-*p*-nitrobenzenesulfonate (Heinrikson, 1971) yields a derivative which chromatographs well in a unique position on SP-400 (between silylated PTH-threonine and PTH-aspartic acid). However this does not appear to be a suitable derivative because S-methylated polypeptides are quite insoluble and difficult to purify.

The S-alkyl proteins are exhaustively dialyzed or passed over Sephadex to remove all salts and reagents. It is especially important to remove urea or guanidine salts since both are deleterious to subsequent sequenator analysis. The salt-free protein (5-10 mg) is dissolved in 0.4 ml of a volatile solvent (e.g., 10-50% aqueous acetic acid) and dried to a film on the lower one-third of the wall of the sequenator cup.

Proteins. PORCINE TRYPSIN (Novo Industri, Copenhagen) was purified by affinity chromatography on insoluble chicken ovomucoid according to Robinson *et al.* (1971).

Three fragments of THERMOLYSIN were derived by cleavage with cyanogen bromide and gel filtration (Titani *et al.*, 1972b). These fragments and certain subfragments derived from them are identified in Table II.

Results

Choice of Buffer for the Coupling Reaction. Quadrol buffer (as described by Edman and Begg, 1967) has a number of desirable characteristics for automated sequencing of poly-

peptides. It is nonvolatile and hence does not cause condensation in the reaction chamber; it is an excellent protein solvent having a pK_a (9) in the desired pH range. However, Quadrol has a number of undesirable characteristics. It is difficult to purify; it is poorly soluble in benzene; and since the buffer salt is practically insoluble in benzene, extraction with ethyl acetate is required to remove the buffer from the protein film. Besides, hydrophobic peptides and short peptides (less than 40 residues) are also extracted by ethyl acetate and their loss represents the most serious practical problem encountered in the use of Quadrol.

Buffers of the same general formula ($RR'NCH_2CH_2N-R''R'''$) have pK_a values in the desired pH range. However, salts of these compounds are generally very insoluble in organic solvents including ethyl acetate. Tertiary amines ($RR'R''N$) are too basic for use if all three substituents are simple alkyl groups. Substituted anilines are not basic enough. Substituted allylamines and benzylamines dissociate in the required range. However, dimethylallylamine buffer, commonly used in manual Edman degradations (Edman, 1970) and in the sequenator (Niall *et al.*, 1969), is quite volatile and must be added repeatedly during the coupling period to maintain the pH in the desired range.

Dimethylbenzylamine (DMBA) appears to be a satisfactory alternative. It has a pK_a of 8.9 (Perrin, 1965), it boils at 180° under atmospheric pressure making it quite nonvolatile in the sequenator, and its acetate, trifluoroacetate, and heptafluorobutyrate salts are quite soluble in benzene, eliminating the need for extraction with ethyl acetate.

Satisfactory coupling requires simultaneous solution of buffer, phenyl isothiocyanate, and polypeptide. To achieve this condition, 1-propanol-water or pyridine-water mixtures were used with varying success. With sperm-whale myoglobin at pH 9.2 in DMBA, consecutive experiments gave repetitive yields of 94% in 40% aqueous pyridine buffer and just over 96% in the aqueous propanol buffer described in Methods. (These yields were based on the recoveries of Val₁₀, Val₁₁, Leu₂, and Leu₁₁ in the myoglobin sequence.) Since an increase in the repetitive yield from 94 to 96% permits the identification of 15–20 additional residues, other factors being equal, the propanol buffer was judged to be superior provided the protein remained soluble.

DMBA was found to be suitable for protein fragments containing 50–150 residues. Since DMBA is semivolatile at 50–60°, it was difficult to avoid condensation of DMBA-heptafluorobutyrate on the walls of the reaction chamber. Static vacuums, no matter how low the pressure, left traces of buffer and acid after each step of the cycle, forming a fog of condensate in the succeeding step. By modifying the sequenator to allow a slow bleed of nitrogen through the cup during the fine vacuum steps, the atmosphere in the reaction chamber was purged and very little condensation occurred.

Preliminary testing indicated that *N*-allylpiperidine has similar properties to DMBA and may also be a suitable buffer.

Thiols. Addition of dithioerythritol (DTE) to the chlorobutane preserved recognizable products corresponding to serine and threonine (Hermodson *et al.*, 1970) and at the same time increased the yields of the other PTH-amino acids to 90–100%.

Since even the most highly purified DTE available interfered to some extent with gas chromatography of the sequenator products, alternate thiols were examined. Mercaptoethyl ether and butanedithiol proved acceptable with regard to stability of PTH derivatives, particularly those of serine and threonine, but these thiols tended to produce increasing

amounts of spurious gas chromatographic peaks during storage, probably by oxidative polymerization. Initial attempts to use highly volatile thiols in the sequenator failed because they evaporated during the automatic sample-drying procedure. This problem was not alleviated by storing the products in the chlorobutane solution containing ethanethiol, since decomposition of the serine and threonine derivatives took place during the aqueous acid treatment. Optimal yields of PTH-serine were obtained by including ethanethiol in both the extracting and cyclizing solutions (see Methods), and this thiol was adopted. In the presence of ethanethiol, serine and threonine yielded additional gas chromatographic peaks. The additional peak for serine appeared near silylated PTH-aspartic acid (illustrated later in Figure 3, cycle 14) and the two additional peaks for threonine appeared near PTH-aspartic acid and after PTH-methionine (Figure 1). Thus the mercaptan may be reacting with degradation products of PTH-serine and PTH-threonine (e.g., PTH- δ -serine and PTH- δ -threonine) producing new stable compounds.*

PTH-proline and PTH-hydroxyproline also react with the silylating agent when any thiol is used. The product of PTH-proline appears between silylated PTH-glutamic acid and PTH-phenylalanine, whereas the product of PTH-hydroxyproline appears between PTH-glutamine and PTH-tyrosine. Small amounts (5–10%) of unsilylated PTH-proline and PTH-hydroxyproline are observed in the sequenator samples. Unsilylated PTH-proline chromatographs near silylated PTH-glycine (illustrated later in Figure 2, cycle 3). The mobilities of other PTH-amino acids are not affected by the inclusion of thiols.

Incomplete Degradation of Proline. When the sequenator was operated at approximately 50°, the degradation of proline residues was incomplete and an overlap of approximately 20% was observed in the succeeding cycles. The yield was not improved by cleaving twice, by changing buffers (DMBA in propanol or pyridine, Quadrol, and *n*-allylpiperidine), by lengthening or shortening the coupling time, or by coupling twice. The problem was minimized by raising the temperature and was aggravated by lowering the temperature in the reaction chamber. At 56–57° the overlap after proline residues was about 5–8%.

Limitation of Size of Polypeptides. The size of the peptide influences the number of definitive degradations. Since polypeptides containing more than 250–300 amino acid residues are poorly soluble in the coupling buffer, significant overlap occurs due to incomplete reaction. Large proteins also generate background quickly since nonspecific cleavage can occur at more sites. Also the larger the protein, the smaller the molar quantity which can be placed into the cup (10 mg appears to be a practical upper limit in most cases). Small peptides (less than 30 residues) tend to be extracted from the cup into the chlorobutane phase, particularly if they are hydrophobic or if the film of peptide is not continuous on the wall of the cup. To prevent this loss, the sample size should be 7–10 mg.

Peptides derived by enzymatic fragmentation of proteins are usually too small for sequenator analysis with the excep-

* One anomaly has been observed with the use of ethanethiol. Occasionally the conversion of PTH-valine and PTH-isoleucine does not go to completion, resulting in a peak appearing prior to any PTH-amino acid in the gas chromatogram and in diminished yield of PTH-valine or PTH-isoleucine (e.g., see Figure 3, cycle 1). Longer conversion times or careful mixing of the HCl solutions usually prevents this anomaly. The occurrence of this phenomenon is erratic but has not interfered with the positive identification of the residue.

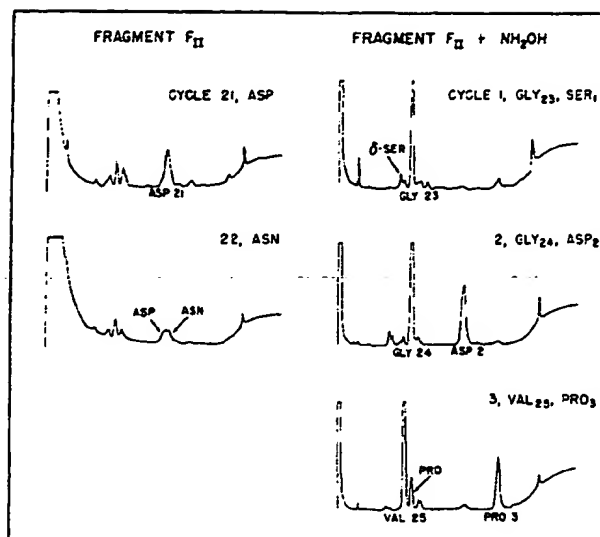


FIGURE 2: Gas-liquid chromatographic analyses of silylated sequenator products derived from the sequence Asp₂₁-Asn-Gly-Gly-Val₂₅ in fragment F₁₁ of thermolysin (residues 226-230, Table II). The number above each chromatogram refers to the degradation cycle under examination. The number beside each amino acid refers to its position in the sequence of F₁₁. (Since F₁₁ represents residues 206-316 of thermolysin, Asp₂₁ corresponds to Asp₂₂₆ of thermolysin.) The vertical scale records the response of the detector in arbitrary units. The tops of large peaks are not always illustrated. The horizontal scale is approximately 20 min/analysis. Left: a sharp drop in product yields is seen between cycles 21 and 22, corresponding to Asp₂₁ and Asn₂₂ in the sequence Asp₂₁-Asn-Gly-. Right: after cleavage of F₁₁ with hydroxylamine (see text), the first three cycles yield both the amino-terminal sequence of F₁₁ (Ser-Asp-Pro) and the internal sequence Gly₂₃-Gly-Val-. (The yield of Ser-Asp-Pro is low because the peptide representing residues 1-22 was partially lost during dialysis.)

tion of fragments obtained by tryptic cleavage of N-acylated proteins. The carboxyl-terminal arginine residues reduce the solubility of such fragments in the extracting solvents.

Three methods of chemical cleavage of proteins yield fragments suitable for sequenator analysis. Fragmentation by cyanogen bromide at methionine residues is both specific and quantitative (Gross, 1967). Cleavage at tryptophan residues has been successfully employed by Niall *et al.* (1971). Cleavage with hydroxylamine at asparaginyl-glycyl bonds (Bornstein, 1969) has been applied successfully on several occasions in our laboratory (*e.g.*, Titani *et al.*, 1972a). Since asparaginylglycine sequences are relatively infrequent, the resulting fragments are quite large.

Low Repetitive Yields. The yield of product at each cycle of the degradation is 95-96% of the product yield in the previous cycle (this has been termed the "stepwise" or "repetitive" yield). Lower yields occur occasionally with particular sequences. For example, during acid cleavage of the preceding residue, certain glutamine residues are partially converted to pyrrolidone derivatives and hence their yield drops 5-10%. The product of this side reaction is stable and does not interfere with subsequent degradation of the unblocked fraction. The low yield of prolyl residues can be largely overcome by raising the temperature to 57° (see above).

Asparaginylglycine sequences also undergo cyclization in acid (Bornstein, 1969) and since the resulting cyclic imide is not susceptible to Edman degradation, the stepwise yield will be low (approximately 50%). However, hydroxylamine

cleaves the imide (Bornstein, 1969) and generates an amino-terminal glycyl residue which can serve as a new starting point for sequenator analysis. These results are illustrated in Figure 2 for cyanogen bromide fragment F₁₁ of thermolysin (see also Table II). It is evident that the repetitive yield falls off sharply between Asp₂₁ and Asn₂₂. When F₁₁ was first exposed to 1 M hydroxylamine, dialyzed overnight against 5% acetic acid, and then subjected to sequenator analysis, the original amino-terminal sequence and the newly formed sequence beginning with Gly₂₃ (Gly₂₃-Gly₂₄-Val₂₅-) were degraded simultaneously (Figure 2). Since the amino-terminal sequence of the intact fragment F₁₁ had already been established, there was no need to separate the products of hydroxylamine treatment prior to analysis.

Degradation of Specific Proteins. THERMOLYSIN. The reliability and efficiency of the sequenator technique were demonstrated by sequence analysis of the cyanogen bromide fragments and subfragments of thermolysin (Titani *et al.*, 1972a,b). The identification of the fragments and the portions placed by the sequenator are given in Table II. The sequences derived by sequenator analyses were confirmed by conventional methods and in no instance did a positive identification by the sequenator fail to be corroborated by conventional procedures. Many of the tryptic peptides from thermolysin could be aligned solely from the sequenator data. These alignments were confirmed both by isolating overlapping peptides (Titani *et al.*, 1972a) and by agreement with crystallographic analysis (Matthews *et al.*, 1972).

The analysis of thermolysin demonstrated several other advantages of the sequenator technique. First, a number of sequences were readily determined which are difficult by conventional methods, *e.g.*, Tyr-Tyr-Tyr-Leu (residues 27-30), Gln-Asp-Asn (residues 31-33), Asp-Asn-Gln (residues 59-61), Gln-Asn-Glu (residues 158-160), and Gln-Asp-Asn (residues 225-227). Second, three residues (140, 237, and 256) were placed which could have been overlooked in conventional analyses because they involve peptide bonds that are relatively resistant to acid hydrolysis (Val-Val and Ile-Ile). Third, chymotryptic and thermolytic peptides of a tryptic peptide of cyanogen bromide fragment F₁ (Val₁₂₁-Lys₁₈₂), which could not be ordered by conventional means, were aligned by sequenator analysis of the first 48 residues of fragment F₁.

Analyses of fragments of thermolysin were greatly facilitated by the introduction of the DMBA buffer. The three cyanogen bromide fragments, F₁₁₁, F₁, and F₁₁ (Table II), contain 120, 85, and 111 residues, respectively. Analysis in Quadrol buffer involved troublesome losses of peptides from the sequenator cup and fewer than 15 degradations of each of these fragments were definitive. In DMBA buffer as many as 48 residues of fragment F₁ and 44 residues of fragment F₁₁HA₂ could be identified (Table II). In addition, 34 residues were placed in a 43-residue tryptic peptide from fragment F₁₁₁ (S-F₁₁₁-T₁ in Table II).

To illustrate the data obtained in an extended analysis, fragment F₁ (see Table II) was subjected to 52 consecutive degradations. Gas chromatograms of 20 of these products are traced in Figure 3 where cycles 1-52 of fragment F₁ correspond to residues 121-172 in the thermolysin sequence (Titani *et al.*, 1972a). The first six analyses demonstrate the purity of the fragment (see footnote 6 regarding the two valine derivatives in cycle 1). In each cycle the peak of a single major PTH-amino acid increases above background levels and decreases in the following cycle. As degradations proceed, a progressive increase in "overlap" is seen (*e.g.*, by contrasting

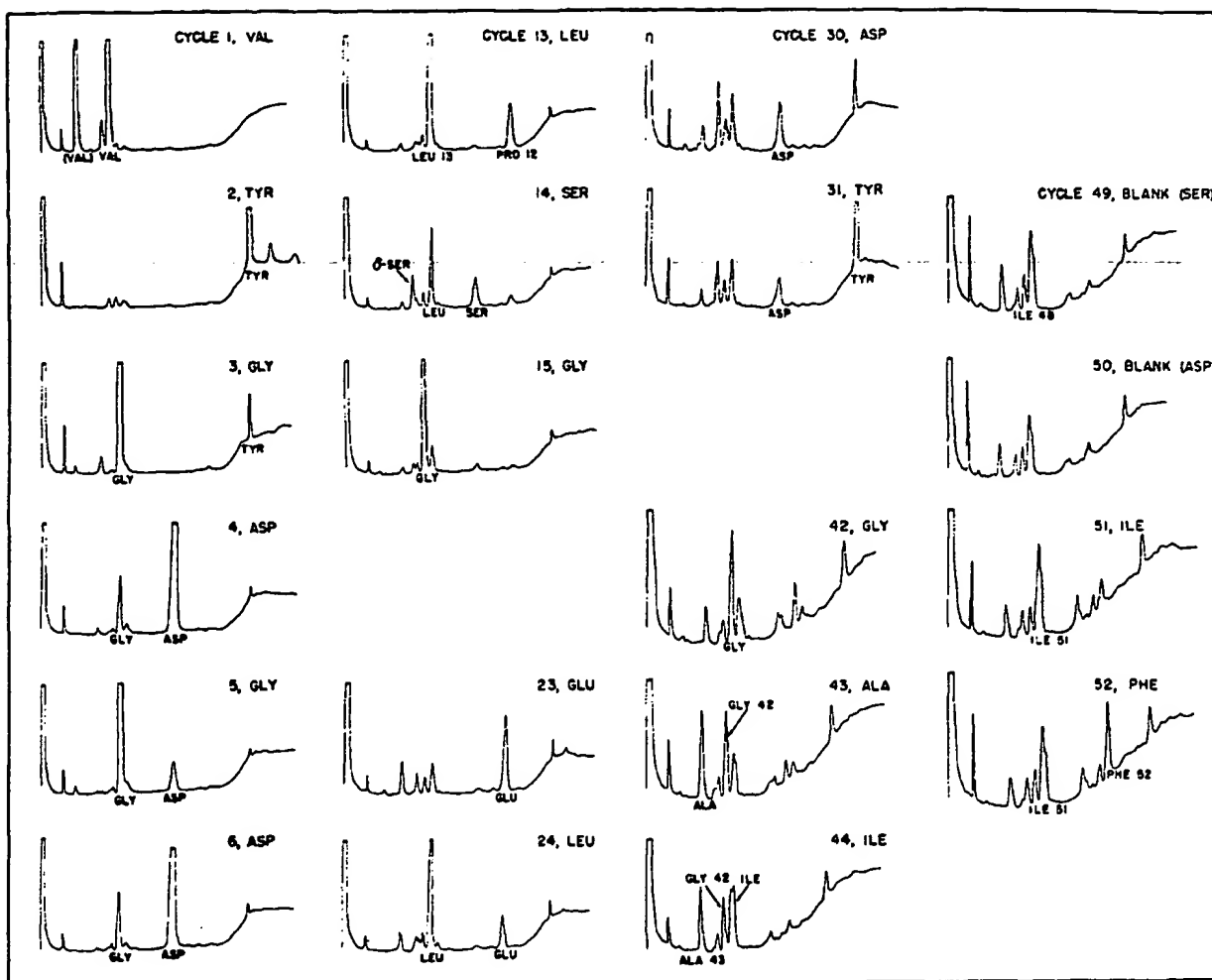


FIGURE 3: Selected gas chromatographic analyses of silylated sequenator products of fragment F_1 of thermolysin (residues 121-205, Table II). The number above each analysis refers to the degradation cycle under examination. (Since F_1 represents residues 121-205 of thermolysin, valine in cycle 1 corresponds to Val₁₂₁ in thermolysin.) The horizontal scale represents approximately 20 min/analysis; the vertical scale (arbitrary units) is the detector response which is electronically attenuated to a greater degree in the earlier cycles than in the later cycles. The tops of large peaks are not always illustrated. The injected aliquot is progressively increased from 3 μ l of a 50- μ l sample in cycle 1 to 6 μ l of a 20- μ l sample in cycle 52. Cycles 41 and 48 (not shown) yielded serine and isoleucine; residues 49 and 50 were identified by other means as serine and aspartic acid.

the very small overlap of Gly₄₁ in cycle 4 with the much larger overlap of Gly₄₂ in cycle 43). In addition, the background level of PTH-amino acids rises relative to the size of the principal product. This is the result of a gradual formation of new amino-terminal residues by nonspecific acid cleavage and a gradual decrease in the principal product arising from incomplete stepwise yield. This aspect of the analysis is somewhat distorted in Figure 3 by the gradual expansion of the vertical scale with increasing cycle numbers. In spite of these problems of interpretation, definitive identifications were possible up to Ile₄₈ (corresponding to Ile₁₃₈ in thermolysin). Neither cycle 49 nor cycle 50 yielded an identifiable product other than the overlap of Ile₄₈. Increases in isoleucine in cycle 51 and in phenylalanine in cycle 52 were the last identifiable products in this analysis.

PORCINE TRYPSIN. Sequenator analysis of porcine trypsin has provided evidence of heterogeneity due to (1) amino acid substitution and (2) an internal split in the polypeptide chain. Both of these phenomena have been previously observed in

proteolytic enzymes: amino acid substitutions in dogfish trypsin (Bradshaw *et al.*, 1970) and in bovine carboxypeptidase A (Pétra *et al.*, 1969) and internal splits in bovine trypsin (Schroeder and Shaw, 1968; Smith and Shaw, 1969; Maroux and Desnuelle, 1969) and in bovine carboxypeptidase B (Reeck *et al.*, 1971). An internal split between residues 131 and 132 of porcine trypsin⁷ was manifest by the simultaneous appearance of a major and minor (5%) peptide sequence when the reduced and carboxymethylated enzyme was subjected to sequenator analysis. The major sequence (β -trypsin) was homologous to the amino terminus of bovine trypsin (11 degradations) and the minor sequence (of α -trypsin) to the sequence of the bovine enzyme starting with residue 132. Gas chromatograms of cycles 10-13 are shown in Figure 4. The major sequence yields in cycle 10 asparagine and the

⁷ Autolytic cleavage at positions 131-132 of bovine trypsin has been characterized by Schroeder and Shaw (1968).

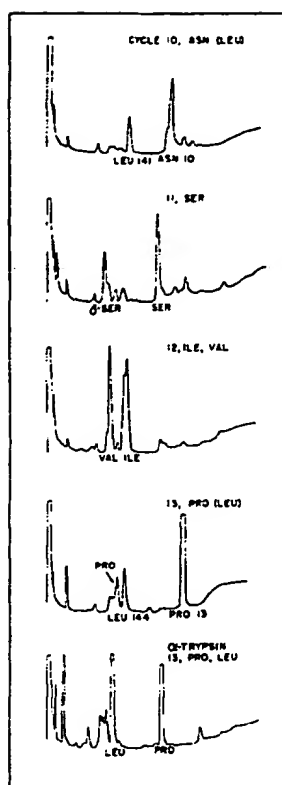
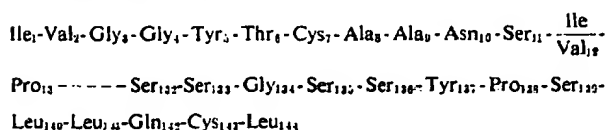


FIGURE 4: Gas-liquid chromatographic analyses of silylated sequenator products from cycles 10-13 of 20:1 mixtures of β - and α -porcine trypsins. The tops of large peaks are not always illustrated. The leucine residues in parentheses are derived from α -trypsin at positions 141 and 144. Cycle 12 reveals two major products, apparently resulting from two allotypic species of trypsin. In the bottom two chromatograms cycle 13 of α -trypsin is compared with that of the 20:1 mixture of β : α (second from bottom). The sequence of porcine trypsin (L. H. Ericsson and M. A. Hermodson, unpublished data) through these areas is:



minor sequence leucine (residue 141). In cycle 11, serine is the major product whereas the corresponding residue of the minor sequence (glutamine) is not detectable. In cycle 12, valine and isoleucine (in a 2:1 ratio) together account for the major sequence in this position, whereas carboxymethylcysteine of the minor sequence was not detectable. In cycle 13, proline is clearly the major product and leucine the minor one.

Recently the porcine enzyme containing the internal split at residues 131-132 (α -trypsin) was separated from the single-chain β form (L. H. Ericsson and M. A. Hermodson, unpublished data). Sequenator analysis of α -trypsin yielded two residues per cycle through 28 degradations. Since the amino-terminal sequence of the intact polypeptide chain had already been determined, the sequence of the 28 residues following residue 131 could be established from these data. Cycles 11 and 12 placed glutamine and carboxymethylcysteine at positions 142 and 143 and confirmed the isoleucine-valine replacement at position 12. Cycle 13 of α -trypsin reveals comparable quantities of Pro_{13} and Leu_{144} (Figure 4).

Discussion

The primary purpose of the automated Edman degradation method is the determination of amino acid sequences. At the present stage of development, 30-50 consecutive residues can be identified and recovered with a stepwise yield of approximately 96%. When the sequenator is in full operation, approximately 90 amino acid residues can be determined per week. Reliability of the method of analysis has been proven by the complete agreement between amino acid sequences of thermolysin derived by conventional methods, on the one hand, and by the sequenator on the other. Sequenator analysis has the additional advantages of rapidity, of direct identification of the amide-containing residues, and of determination of repetitive residues which can present difficulties in conventional sequence determinations (e.g., Val-Val and Tyr-Tyr).

The major advantage of sequenator analysis is its ability to determine extended amino acid sequences (30-50 residues) in a single operation. This procedure eliminates the necessity for extensive fragmentation of the protein prior to sequence analysis and consequently reduces the number of overlaps necessary for aligning the fragments. In the specific case of thermolysin (Titani *et al.*, 1972a) the conventional approach involved the isolation and structural analysis of 173 small peptides from enzymatic digests. In contrast, sequenator analysis of six large fragments provided 60% of the total structure. Although in this case sequenator analysis served to confirm the structure previously established by conventional methods, sequential degradation of fewer and larger fragments would have provided a better starting point for the complete structure analysis. In the case of amyloid protein A containing 76 amino acid residues (Hermodson *et al.*, 1972), sequenator analysis of the whole protein and of a large cyanogen bromide fragment provided the sequence of all but the six carboxyl-terminal amino acid residues.

In addition to the primary purpose of accelerating the determination of amino acid sequences, the sequenator has proven useful in solving other problems usually encountered in the structural analysis of proteins. For instance, in the past a crucial test for purity of a protein has been the demonstration of a single amino-terminal residue by the quantitative cyanate method (Stark, 1967). A few degradations in the sequenator can provide the same information with a smaller quantity of protein and in addition can identify succeeding residues. This additional information eliminates the possibility that the protein in question may contain a mixture of other proteins having the same amino-terminal residue.

However, single chain proteins may yield multiple sequences in the sequenator if internal peptide bonds have been cleaved prior to sequence analysis. The two most prevalent causes of peptide-bond cleavage are limited proteolysis and instability of certain peptide bonds toward acids. The following examples show the usefulness of sequenator analysis in locating the internal split and in relating the simultaneous products of sequenator analysis to the amino acid sequence of the original, single polypeptide chain. (1) Sequenator analysis of human amyloid protein A (Benditt *et al.*, 1971) revealed two residues per cycle in a yield ratio of approximately 7:1. The major sequence was H-Arg-Ser-Phe-Phe-Ser-Phe-Leu-Gly-, the minor sequence H-Phe-Ser-Phe-Leu-Gly-. The two sequences were related in that the minor sequence lacked the amino-terminal tripeptide Arg-Ser-Phe. (2) Analysis of nerve growth factor from mouse submaxillary glands (Angeletti and Bradshaw, 1971) gave two residues per

cycle in approximately equal yield (M. A. Hermodson, R. H. Angeletti, and R. A. Bradshaw, unpublished data). No unique sequences could be deduced from the residue yields in each cycle until a repetitive pattern was observed beginning with the ninth cycle by pairing the residues in cycles 1 and 9, 2 and 10, 3 and 11, etc. These data showed that approximately one-half of the preparation of nerve growth factor lacked the first eight amino acid residues. (3) Heterogeneity due to the acid lability of aspartyl-proline bonds (Piszkiewicz *et al.*, 1970) was clearly evident in the carboxyl-terminal cyanogen bromide fragment of thermolysin (residues 206-316). Sequenator analysis revealed two minor sequences comprising approximately 5-10% of the major sequence. Both minor sequences started with proline, one appearing at cycle 3 of the major sequence and the other at cycle 9 (corresponding to residues 208 and 214, respectively).

In several instances limited proteolysis of internal peptide bonds has resulted in a protein which is apparently pure by the usual electrophoretic and hydrodynamic criteria but which yields multiple sequences in sequenator analysis. The newly established amino-terminal residue provided an opportunity for the determination of extended, internal amino acid sequences. Bovine carboxypeptidase B is a case in point: it yielded an additional 26 amino acid residue sequence in the interior of the molecule and served to strengthen the hypothesis of homology with carboxypeptidase A (Bradshaw *et al.*, 1969; Reeck *et al.*, 1971).

Sequenator analysis of whole proteins has facilitated the proof of homology. For instance, comparison of the first 20 amino-terminal residues of bovine, dogfish, and lungfish trypsinogen has established their homologous relationships (Hermodson *et al.*, 1971). Smithies *et al.* (1971) have made similar use of sequenator analysis to compare light chains of Bence-Jones proteins; Rochat *et al.* (1970) have compared various toxins; and Niall *et al.* (1971) have examined related hormone structures. In our laboratory the homology between amyloid proteins of man, monkey, and Pekin duck (Benditt *et al.*, 1971; L. H. Ericsson and E. P. Benditt, unpublished observations) has been established by analysis of the whole proteins in the sequenator. The strength of the argument for homology based on sequenator data lies in the direct comparison of common regions in the respective polypeptide chains.

The direct determination of amino acid sequences in the sequenator has also given ready evidence for amino acid substitutions in proteins. A protein preparation obtained from pooled animal tissue may contain a mixture of allotypes which would complicate the assembly of sequence information. For instance, bovine β -lactoglobulin and bovine pancreatic carboxypeptidase A each occur in the form of two allotypes of comparable gene frequencies (Ashaffenburg and Drewry, 1957; Pétra *et al.*, 1969). If the allotypic change occurs in a portion of the sequence accessible to the sequenator, such a mutation is readily and unequivocally identified. In the case of porcine trypsin, such a mutation apparently has occurred in position 12 (Figure 4).

Sequenator analysis can resolve the number and identity of protomers in an oligomeric protein. In one specific case examined in this laboratory (aspartokinase-homoserine dehydrogenase I)^a sequence analysis of the whole protein clearly established that the four subunits are identical throughout

the first eight amino-terminal residues, suggesting that the subunits are either identical or homologous.

The examples given in this report are illustrative of the variable applications of sequenator analysis in determining the covalent structure of proteins. The versatility of the method is evident from the examples given, but further improvements in the operation of the method, particularly in the number of consecutive degradations, will further enhance the usefulness of the method in the direct determination of the amino acid sequence of yet larger protein fragments.

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^a This enzyme was provided by Professor Georges Cohen, Institut Pasteur, Paris, and the detailed results will be communicated separately by his laboratory.

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Mechanism of Bovine Prothrombin Activation by an Insoluble Preparation of Bovine Factor X_a (Thrombokinase)[†]

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ABSTRACT: Sepharose-factor X_a (TK-resin) has been prepared and used for the investigation of the generation of thrombin from a purified bovine prothrombin. Thrombin generation was followed by assay for clotting activity and by disc gel electrophoresis for reaction products. Under the conditions employed (25% w/v sodium citrate, pH 6.5, 24°) prothrombin activation is essentially complete (80–100%) when compared to a two-stage activation assay. Autocatalysis could not be demonstrated either by withdrawing TK-resin before complete activation or by adding activation product to fresh prothrombin. Prothrombin in the presence of 25% sodium citrate alone, in the presence of activation products, or in the presence of thrombin does not generate clotting activity; however, prothrombin in the presence of TK-resin, with or without sodium citrate, will produce clotting activity. The complex activation pattern as observed by disc gel electrophoresis is best explained by two activation pathways: one initiated

by factor X_a, and the other by thrombin. The factor X_a pathway leads from prothrombin to a single-chain molecule, P₁, plus an intermediate, F_X, which (in the presence of thrombin) rapidly decomposes. The thrombin-initiated pathway leads from prothrombin to a fragment, F_A, and an intermediate, P₂. The latter is further broken down to two single-chain molecules: a fragment F_B and an intermediate P₃. Both pathways appear to converge to the same thrombin precursor, P₃. Thrombin forms from P₃ only in the presence of factor X_a. The double-pathway mechanism was tested by reacting prothrombin (1) with TK-resin in the absence of thrombin (in the presence of 0.0079 M DFP) and (2) with thrombin in the absence of TK-resin. Results from these experiments and the estimated molecular weights of the intermediates are consistent with the proposed mechanism. The implications of a double pathway in physiological prothrombin activation are discussed.

Central to the phenomenon of hemostasis is the activation of prothrombin to thrombin, which catalyzes the polymerization of fibrinogen and thus forms the definitive blood clot. Despite its importance and an intensive investigative effort over many years the activation mechanism remains incompletely understood. This is due in part to the difficulty

in preparation and stabilization of the zymogen and its intermediates and to the apparent complexity of the pathway. The chemistry of prothrombin and thrombin and a discussion of proposed activation mechanisms have been reviewed recently (Magnusson, 1971). As discussed by Magnusson it is generally accepted that bovine prothrombin, a single-chain plasma glycoprotein without clotting activity, of apparent molecular weight 68,000–74,000, liberates a double-chain serine protease with clotting activity, having a molecular weight of 33,700–40,000. From these facts one may assume that the activation mechanism entails (1) at least two proteolytic cleavages of the zymogen to yield the double-chain enzyme, and (2) the liberation of a large piece(s) of the prothrombin molecule.

Although it is not certain how prothrombin is activated *in vivo*, in the laboratory the conversion of prothrombin to

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